Herbicide resistance in Black-grass (*Alopecurus myosuroides* Huds.)

Thesis

Herbicide resistance in Black-grass

(*Alopecurus myosuroides* Huds.)

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Declaration

This thesis was composed by the author and is a record of work carried out by her on an original line of research. All sources of information are shown in the text and listed in the references; all help given by others is indicated in the acknowledgements.

None of this work has been presented in any previous application for any degree or qualification.

Signed ..... (Lucy J Milner)

Abstract

Herbicide resistance in *Alopecurus myosuroides* (Huds). (Black-grass).

Herbicide resistant grass weeds are a growing problem throughout the UK with *Alopecurus myosuroides* Huds. (black-grass) considered a major problem in winter cereals. Black-grass control is hindered by the presence of populations resistant to herbicides. Research indicates that resistance in black-grass is due in part to enhanced metabolism involving glutathione *S*-transferases (GSTs) and that increased activities of these enzymes may confer resistance in this species. The work described in this thesis has characterised resistance in black-grass and examined the role GSTs play in herbicide resistance with respect to herbicide application and timing.

Characterisation of herbicide resistance in three black-grass populations tested against isoproturon, fenoxaprop-P-ethyl, clodinafop-propargyl, sethoxydim, flupyrsulfuron-methyl and AC210 in the glasshouse revealed that the commercially available population, Herbiseed, may be used as a standard susceptible reference population when testing unknown populations. Novel resistance ratings were applied to Herbiseed for future reference.

A 2 year study was performed to investigate glutathione *S*-transferase activity in five UK black-grass populations from field sites situated in the East Midlands. Findings indicate there is a natural elevation of endogenous GST activity in response to black-grass growth and development and natural environmental changes from winter to spring. Clear correlations between GST activity, temperature, solar radiation and sunshine hours have been observed. It is proposed that increasing GST activity is required as part of an antioxidant defence system until tillering (GS30) has ceased. It is speculated that this endogenous change in enzyme activity with plant development in the field contributes to reduced efficacy of some graminicides applied in the spring.

Further investigation in a controlled environment focused on the effect of temperature on plant growth and antioxidant status of resistant and susceptible black-grass. Results indicated that temperature has a developmental and metabolic effect on the growth of resistant black-grass plants, which may be critical in the response of plants to herbicide treatment. Increased temperature was accompanied by a natural elevation in endogenous GST activity in resistant plants and changing temperature increased the concentration of antioxidants. It is speculated that these endogenous responses are part of a natural mechanism of acclimation to environmental change in resistant plants of this species and provide protection against subsequent stress such as herbicide treatment.

In conclusion, it is postulated that the antioxidant system of black-grass plants is vital for survival under normal plant growth and development and climatic conditions. It is speculated that these endogenous responses are part of a natural mechanism of acclimation to environmental change whilst supporting normal plant development, suggesting that GSTs have direct cytoprotective activity. These observations lend further weight to the suggestion that the development of resistance in black-grass is in part due to evolution and elevation of GST activity. It is speculated that in striving to achieve maximum herbicide efficacy in resistant black-grass populations, the period of environmental change from autumn to winter as temperature decreases, in combination with smaller growth stages of plants would be the best time for graminicide application for black-grass control.

Dedication

This thesis is dedicated to my parents – David and Sheila

Thank you for all your love and support over the years

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PAPERS PUBLISHED PRIOR TO COMPLETION OF THIS THESIS

Milner L J, Reade J P H and Cobb A H (1999). An investigation of glutathione *S*-transferase activity in *Alopecurus myosuroides* Huds. (black-grass) in the field. *Proceedings of the Brighton Conference, 1999, Weeds,* **1**, 173-178.

Milner L J, Belfield J L, Reade J P H and Cobb A H (1999). An investigation of the detoxification of active oxygen species in black-grass (*Alopecurus myosuroides*) plants susceptible and resistant to herbicides. *Proceedings of the Brighton Conference, 1999, Weeds,* **2**, 561-562.

Milner L J, Reade J P H and Cobb A H (2001). The effect of temperature on glutathione *S*-transferase activity in *Alopecurus myosuroides* Huds. (black-grass). *Proceedings of the Brighton Conference, 2001, Weeds,* **1**, 345-348.

Milner L J, Reade J P H and Cobb A H (2001). Developmental changes in glutathione *S*-transferase activity in herbicide-resistant populations of *Alopecurus myosuroides* Huds. (black-grass) in the field. *Pest Management Science,* **57**, 1100-1106.

Reade J P H, Milner L J and Cobb A H (2001). Herbicide resistance in *Alopecurus myosuroides* (black-grass): field testing and population plasticity. *Proceedings of the Brighton Conference, 2001, Weeds,* **2**, 577-582.

List of Abbreviations

ALS	Acetolactate synthase inhibitors
ACCase	Acetyl CoA carboxylase
a.i.	Active ingredient
AOS	Active oxygen species
ACP	Advisory Committe on Pesticides
AOPP	Aryloxyphenoxypropionates
APX	Ascorbate peroxidase
AsA	Ascorbate
BSA	Bovine serum albumin fraction V
CDNB	1-chloro-2, 4-dintrobenzene
CHD	Cyclohexanediones
CON	Control
CP	Clodinafop-propargyl
CTU	Chlorotoluron
CV	Coefficient of variation
°C	Degrees centigrade
d	Days
DasA	Dehydroascorbate
dat	Days after treatment
DHAR	Dehydroascorbate reductase
DTNB	5,5'-dithio-bis(2-nitrobenzoic acid)
DTPA	Diethylenetriaminepentaacetic acid
EM	Enhanced metabolism
FE	Fenoxaprop-P-ethyl
FPM	Flupyrsulfuron-methyl
FR	Field rate
g	Grams
GPX	Glutathione peroxidase
GR	Glutathione reductase
GSH	Glutathione
GST	Glutathione *S*-transferase
GSSG	Glutathione disulfide
h	Hour
HRAC	Herbicide Resistance Action Committee
H_2O_2	Hydrogen peroxide
IPU	Isoproturon
max	Maximum
MDAR	Monodehydroascorbate reductase
min	Minutes
ml	Millilitres
mm	Millimetres
MOA	Mode of action
m/m	Mass/Mass
OM	Organic matter
O_2	Oxygen
P450	Cytochrome P450 enzymes
PCR	Polymerase chain reaction
Post-em	Post-emergent
PPO	Protoporphyrinogen oxidase
Pre-em	Pre-emergent
PSII	Photosystem II

PVPP	Polyvinylpolypyrrolidone
rcf	Relative centrifugal force
S	Sulphur
SDS	Sodium dodecyl sulphate
sec	Seconds
SETH	Sethoxydim
SOD	Superoxide dismutase
TS	Target site
WRAG	Weed Resistance Action Group

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CHAPTER ONE

INTRODUCTION

1.1. GRASS WEEDS IN UK AGRICULTURE

In 1977, Holm, Plucknett, Pancho and Herberger compiled an inventory of the world's worst weeds affecting agriculture and identified 18 weed species as the most problematic at that time. Of these 18, 10 were members of the *Poaceae*. For the purpose of this thesis, a weed is defined in accordance with the European Weed Research Society as, 'any plant or vegetation interfering with the objectives of people' i.e. a plant growing where it is not wanted (Mortimer, 1990). At present, in a global context, only 250 plant species are considered problematic enough to be termed 'weeds'. Of these 250, 70% are found in 12 plant families, with over 40% alone being members of the *Poaceae* and *Compositae*. Grass weeds currently account for 25% of the world's principal weeds (Holm, Plucknett, Pancho and Herberger, 1991; Cobb, 1992; Holm, Doll, Holm, Pancho and Herberger, 1997).

For centuries, grass weeds have been regarded as of little importance compared to broad-leaved weeds in arable crops. Broad-leaf weed control has been practised since 8000BC, when *Galium aparine* (cleavers) appeared and has been relatively straightforward. In contrast, interest in grass weeds (both annual and perennial) such as *Alopecurus myosuroides* (black-grass) and *Avena* species (wild oats) in cereals, has only surfaced as recently as the 1950's (Gwynne and Murray, 1985). Incidence of problematic grass weeds has increased dramatically in the last thirty years. This is due to changes in agricultural practices in the form of reduced cultivation and increased reliance on chemical control together with general mechanisation, reduced crop rotations and adoption of monoculture, as well as the increased use of fertilisers. Grass weeds cause substantial yield losses and damage during early stages of crop growth through competition and stunt their later development. They also act as hosts or shelters for numerous pests such as aphids, frit flies, nematodes and wheat bulb flies, and diseases such as mildew, rusts and take-all.

Except for the *Poaceae*, very few other monocotyledons (*Cyperaceae, Lilaceae*) are of any significance as weeds of cultivated land (Behrendt and Hanf, 1979).

As grass weeds and cereals are both members of the *Poaceae*, they parallel each other in morphology and physiology. Grass weeds could be postulated to compete more effectively with cereals than broad-leaf weeds and research confirms this. Baldwin (1979) indicates mean yield responses after the removal of black-grass and wild oats of 10-20%. In contrast, Snaydon (1982) reports similar trials involving broad-leaved weeds revealing a figure of just 2%. Grass weeds are undoubtedly the biggest problem associated with cereal crops. The UK problem was evident in the early 1970's and has substantially intensified since. Agricultural intensification through the adoption of continuous cereal growing regimes has led to several prominent grass weed species establishing themselves, including black-grass, wild oat species, *Bromus* spp (bromes), *Elytrigia repens* (couch grass), *Lolium multiflorum* (Italian rye-grass) and *Poa* spp (meadow grasses) (Siddall and Cousins, 1982; Chancellor and Froud-Williams, 1986).

The most problematic grass weeds in the UK are currently black-grass, wild oat species and Italian rye-grass (Moss, Clarke, Blair, Culley, Read, Ryan and Turner, 1999), which are highly competitive, invasive weeds within cereal crops. Grass weed control is predicted to become increasingly important, with the probable increase in intensification of winter wheat, favouring annual grass weed occurrence. This is of significant importance as winter wheat is one of the few crops that the UK grows competitively in international markets

1.2. *ALOPECURUS MYOSUROIDES* HUDS. (BLACK-GRASS)

Black-grass is a member of the *Poaceae*, which forms one of the largest plant families in the world, comprising 610 genera and approximately 10,000 species (Langer and Hill, 1991). The genus *Alopecurus* with more than 20 species within it can be found distributed throughout Europe and Asia. Some species are present in North and South America but these have most probably been introduced. *Alopecurus myosuroides* Huds. (black-grass or slender foxtail) is the only member of the *Alopecurus* genus found as a common arable weed (Behrendt and Hanf, 1979). Black-grass populations within the UK can also be separated into distinct biotypes with respect to herbicide resistance. These biotypes are normally named after their place of origin e.g. Rothamsted, Hertfordshire and Peldon, Essex.

Evidence indicates that black-grass is not a weed of recent origin and that it was known in Neolithic times (4000-2000 BC), as a weed of emmer and one-grained wheat (Behrendt and Hanf, 1979). Black-grass is probably native to the Mediterranean region of Europe and of Western Asia through to India, and introduced elsewhere (Naylor, 1972a) but is now present globally as indicated in Table 1.1. Within the UK, black-grass is a widespread problem, with infestations found mainly in central and eastern counties, spreading into northern counties, with incidence on a constant increase.

1.2.1. Characteristics

Black-grass is a common arable and wasteland weed. It is a tall, erect, tufted or sometimes solitary annual grass, which is propagated solely by its own seeds. Plants possess a shallow root system and have distinctive green or purplish leaf sheaths. They typically range between 30-90cm in height and possess a single compact flowering head for each reproductive stem (Naylor, 1972a; AgrEvo, 1997a). Table 1.2. shows the main

characteristics of black-grass and Figure 1.1. illustrates its seed cycle, identifying the sequence of events which maintain its presence in the field and potential in the soil.

Table 1.1. Global occurrence of black-grass

(Source: Holm *et al.*, 1991)

S	P	C	X	F
England	Belgium	Austria	Afghanistan	Australia
Germany	India	Iraq	France	
Hungary	Iran	Israel	Greece	
Italy	Lebanon	Netherlands	Jordan	
	Sweden	Commonwealth of Independent States	Norway	
		Turkey	New Zealand	
			Pakistan	
			USA	

KEY: **S** – Serious weed; **P** – Principal weed; **C** – Common weed

X – Present as a weed (the species is present and behaves as a weed, but its rank of importance is unknown).

F – Flora (the species is known to be present in the flora of the country, but confirming evidence is needed that the plant behaves as a weed).

Once established, plants are relatively tolerant of high moisture, hence black-grass is frequently associated with heavy soil or lighter compacted land with poor drainage. However, this is not a pre-requisite for its occurrence as there is no correlation between soil type and distribution, illustrated by the fact that infestations have also been recorded on lighter, well drained soils (Naylor, 1972a; Moss, 1981). As early as 1913, Brenchley suggested that black-grass might well be more directly adapted to a cropping system than a particular soil type.

Table 1.2. Characteristics of black-grass

Germination pattern	Episodic. Main – Sept, Oct, Nov Secondary – Early spring, March and April 80% in autumn	(Naylor, 1972a) (Mortimer, 1990) (Thurston, 1964)
Germination pre-requisites	Temperature range 10-25°C Optimum 15°C No germination <3°C or >30°C Light and high moisture Emergence depth – 90% from top 2.5 cm (range 0-5cm)	(Barralis, 1968) (Koch, 1968) (Froud-Williams, 1985) (Naylor, 1970) (Thurston, 1964).
Tiller production	Up to 150 per plant	(Gwynne and Murray, 1985)
Reproduction	Allogamous Dispersal unit – spikelet Self-fertilisation possible	(Naylor, 1972a)
Seed production	1->1000 heads m^{-2} 20-300/head dependant upon plant age and no. of tillers	(Moss, 1981) (Naylor, 1972a)
Seed shedding period	Late June - Late August Peak – late July	(Moss, 1981 and 1983)
Seed viability	Variable - 43-76% (lower in early and late shed seeds)	(Naylor, 1972b) (Moss, 1981 and 1983)
Seed longevity	48-72 months (varies)	(Mortimer, 1990)
Dormancy	Some seeds germinate immediately after shedding Most have a short, innate and sometimes enforced dormancy	(Barralis, 1968) (Naylor, 1972a) (Froud-Williams, 1985)
Loss of viable seeds from seedbank p.a. (all causes)	60-80%	(Mortimer, 1990) (Moss, 1997)
Seedlings established p.a. (% viable seedbank)	In surface soil (0-5cm) range - 10-40% for new seeds 10-65% for old seeds buried	Moss (1990a)

Black-grass is now most commonly associated with winter-sown crops, especially cereals, due to its germination patterns and as a result of technological changes in the way that winter cereals are grown, making the agricultural activities of man responsible for its distribution and abundance as a species. This includes factors such as minimal cultivation and early sowing of crops. These increasing trends in cereal production result in viable seed being left near the soil surface where it can successfully germinate and establish (Moss, 1979).

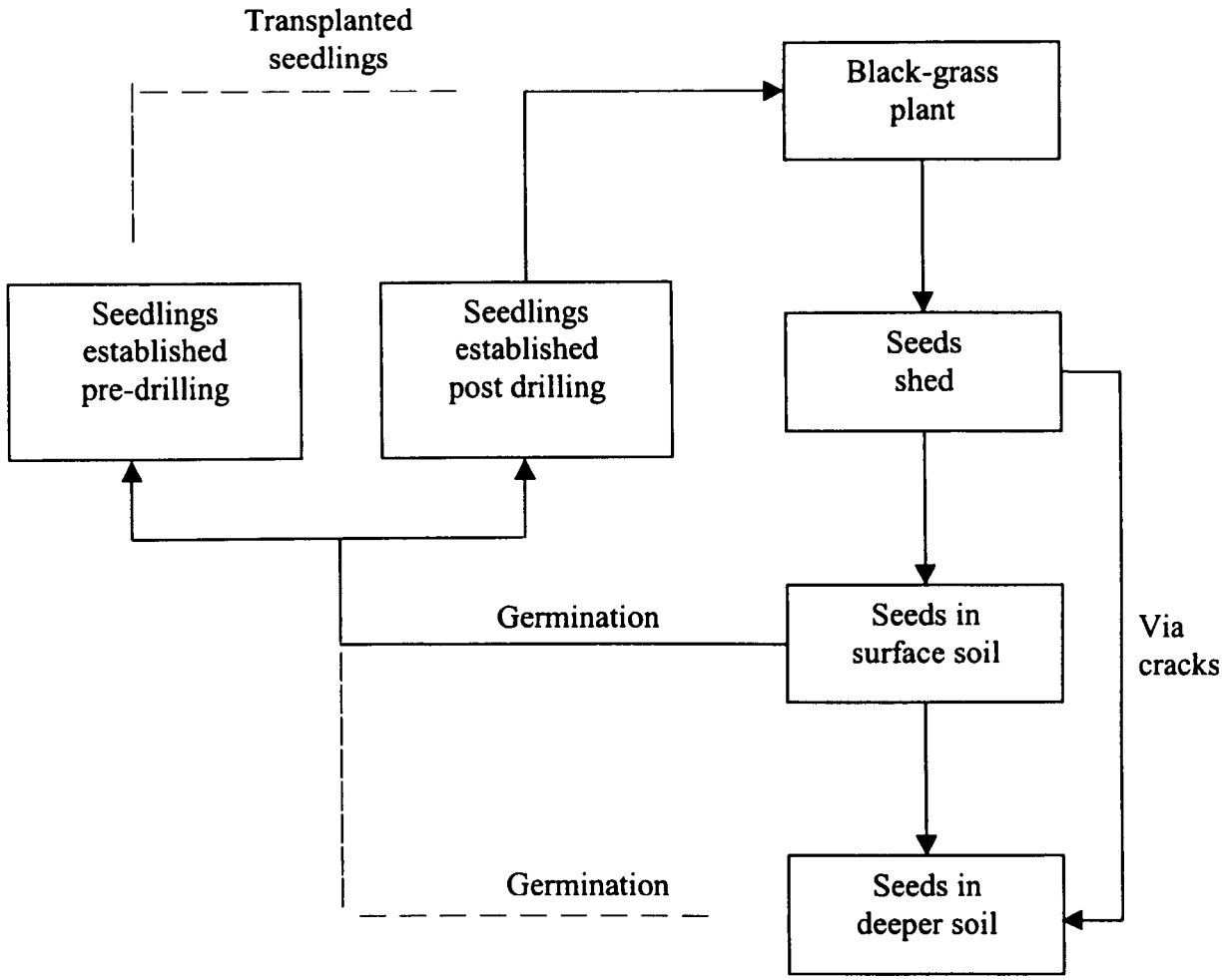


Figure 1.1. Seed cycle of black-grass (*Alopecurus myosuroides* Huds.)

(Source: Moss, 1981)

1.2.2. The agricultural importance of black-grass.

Naylor (1972a) predicted that the increasing popularity of cereal monculture would result in a direct rise in the economic importance of black-grass, with severe infestations occurring with increasing frequency. This is supported by Moss (1981) who indicates that as the area of winter sown cereals increases in the UK and crops are grown successively on the same land, conditions increasingly favour black-grass infestation. Black-grass is now said to infest approximately 700,000ha of winter-sown crops in England (Orson and Harris, 1997).

Black-grass plants and cereals grow in a highly competitive situation and compete directly for space, nutrients and light. Competition is by direct replacement (Naylor, 1972b; Moss, 1987a). Crop and weed emergence often coincides in the autumn. Once established, black-grass plants develop at the same or a slightly slower rate than the crop. However, between April and June black-grass development becomes much more rapid, thus crop and weeds compete against each other at a time that coincides with the period of rapid crop growth. Black-grass is exposed to competition both with the crop and (like the crop) from other weeds. Thus, crop competition goes some way to determining the extent of weed infestation within the crop (Wallgren and Aamisepp, 1977; Moss, 1981; 1987a;). However, research indicates that black-grass infestations that appear insignificant in autumn and which have relatively little effect on crop growth prior to April, can have detrimental effects on cereal grain yields (Moss, 1987a).

The main justification for controlling black-grass infestations is the potential loss of yield. Although crop plant populations are not affected by the presence of black-grass, densities of 250-500 black-grass plants m^{-2} can decrease cereal yields by approximately 45%. More severe infestations can reduce yields by up to 66%. Black-grass achieves this by reducing crop head densities and number of grains per ear, albeit with little or no effect on thousand grain weights (Moss, 1981; 1987a). As well as the potential loss of yield, black-grass can also cause other detrimental effects to cereals. At harvest, black-grass can cause both increased harvesting difficulty associated with combining green plant material and a higher grain moisture content due to the presence of grain contamination. Black-grass is also known to carry the take-all fungus (*Gaeumannomyces graminis*) and to counteract any beneficial effects of a rotation in containing the disease. In addition it is a secondary host and a major source of inoculum of ergot (*Claviceps purpureum*) in wheat (Gair, Jenkins, and Lester, 1991). These problems can cause extra cost and/or loss of revenue for cereal farmers. Several studies have been carried out observing competition between wheat and

black-grass, which suggest that long term black-grass control depends upon reducing seed return and minimising seed production, thus reducing populations, especially in reduced cultivation systems (Moss, 1981; 1983).

1.3. BLACK-GRASS CONTROL MEASURES

Moss (1981) indicates that the use of a combination of weed control measures should improve overall suppression of black-grass and knowledge of factors influencing populations in the field enables integrated control strategies to be developed. Most weed control measures aim to prevent crop and weed competition particularly in ensuring that the crop can grow unhindered by competition from early April. There is no shortage of advice on black-grass control. Herbicides have been the main method of controlling black-grass for many years as they cause plant death and suppress growth and seed production of survivors. Moss and Cussans (1985) estimate the control required from herbicides to maintain a static weed population to be 50% for ploughed land and 88% on direct drilled land. Until 1992, one of the main weapons for black-grass control was straw burning after harvest, which destroyed up to 70% of seed on the soil surface (Moss, 1980). However, since it was banned, the spread of resistant populations and restrictions on the use of isoproturon (IPU), a total approach for the control of black-grass has had to be adopted as opposed to just a herbicide based approach.

In recent years, chemical control has been coupled with non-herbicidal methods towards an integrated control strategy in an attempt to suppress black-grass populations whilst taking into account economics, the environment and resistance risks. Cultural control methods have a large role to play and they also prepare the way for fully effective use of a herbicide programme (autumn and/or spring) and together can produce an effective anti black-grass strategy. Table 1.3. shows the current recommended cultural control methods for black-

grass, many of which are used in conjunction with an effective herbicide programme. Trials have shown that ploughing can reduce black-grass populations in winter wheat by an average of 63% relative to non-inversion tillage. Delayed drilling of winter wheat by three weeks can also reduce populations by an average of 42% (Moss, 1997). Chemical control options for black-grass are based upon the use of residual, soil-acting herbicides e.g. IPU, contact foliar acting compounds e.g. clodinafop-propargyl, or increasingly, products which combine 2 modes of action (MOA) having contact and residual activity e.g. clodinafop-propargyl and trifluralin.

Isoproturon, as a residual herbicide in its own right or as a basis of tank mixes, has been successfully used in cereals for over 25 years, especially for autumn grass-weed control in winter cereals. However in the early 1990's, IPU was detected in surface water levels, above those set by the EC directives (Blair and Orson, 1993). The government's Advisory Committee on Pesticides (ACP) conducted a review on the use of IPU in the UK resulting in a number of restrictions being placed on its usage. Pre-emergence (pre-em) use of IPU is no longer permitted on wheat and barley and total active ingredient (a.i.) usage is limited to 2500g a.i. ha^{-1} per crop per year, although there is a certain degree of flexibility in the way that this can be applied. The ACP also investigated the potential for restricting the amount of IPU applied in any one season to 1500g a.i.ha^{-1}. The IPU stewardship programme guidelines recommend this where possible, using mixtures or sequences with other herbicides (Monsanto, Rhône-Poulenc, Ciba and Touche Ross Agriculture, 1995 and Whitehead, 2001). Studies using reduced rates of IPU have been carried out, but control of black-grass remains optimum at 2500g a.i. ha^{-1} (Ayres and Smith, 1985; Clarke, 1987). The reduction proposals and recommendations have serious implications for the use of IPU for black-grass control in the UK due to the lack of satisfactory control. In addition there is a risk of accelerating the build-up of herbicide resistant black-grass with the increased use of chemicals despite their better environmental profiles, notably

aryloxyphenoxypropionate (AOPP) or cyclohexanedione (CHD) herbicides (Moss and Clarke, 1995).

Table 1.3. Current cultural control methods recommended for black-grass.

(Source: Moss, 1997)

Cultivations	Annual or rotational ploughing to >5cm depth Crop harrowing, inter-row cultivation All reduce seed return
Crop rotation	Spring sown crops Inclusion of grass leys Reduces herbicide usage and extends range of a.i's Future- GMO crops allowing non-selective herbicide use
Set a side/fallowing	Opportunity to reduce seed populations Sow plant cover and/or use of non-selective herbicides in May/early June Patch spray/cutting, roguing in small populations
Stubble hygiene	Destroy weed seedlings prior to drilling using non-selective herbicides
Delayed drilling	Allows more seedlings to emerge prior to drilling to spray off.
Crop competition	Competitive crops are better able to suppress weeds e.g. by increasing seed rates, more competitive crops/varieties, narrower row spacing, good seedbeds, improved drainage
Prevention of seed return	Cutting or spraying off weed patches using a non-selective herbicide Roguing low weed populations
Avoidance of introduction and spread of resistant seeds and plants	Avoid contaminated seed Minimise dissemination of seeds and plants through combines, cultivation equipment, straw or manure Contain resistant weeds in one area e.g. plant cultural and harvesting operations to contain them Finish combining in a weed free area to minimise spread of weed seed from field to field

The review of IPU coincided with the introduction of herbicide formulations with a combination of contact and residual activity. These products have been developed in response to dissatisfaction with the consistency of black-grass control achieved by autumn applied residual herbicides and the increasing threat of herbicide resistance in the UK. Spring control of black-grass has traditionally been achieved with the use of contact

herbicides. However, this option has also proved unsatisfactory due to resistant populations, or well established mature autumn-germinating plants. Delaying control until the spring also allows black-grass to compete with the crop, resulting in yield and financial penalties. Products with combined MOA have been designed to overcome these issues, offering a more broad-spectrum approach to autumn weed control. They differ from older chemistry in that their recommendations for use are based on the growth stage of the weed, as opposed to the crop. These products are now used in combination with residual products in the autumn to maximise black-grass control where possible (Monsanto *et al.*, 1995). Within cereals, sequences of pre- and post-emergence (post-em) sprays are used, sensitising black-grass, to make it more susceptible to later autumn sprays at GS12/13 stage. It is widely acknowledged that autumn applications of herbicide to black-grass tend to be more effective than spring applications as plants are less mature in growth stage and more susceptible to herbicides (Baldwin, 1981). Several new potential black-grass herbicides are currently being developed by agrochemical manufacturers which, if approved, will be available on the commercial herbicide market within the next few years. Table 1.4. gives a comprehensive list of herbicides which are currently approved for the control of black-grass in various cropping systems.

The first experimental chapter of this thesis explores the activity of 6 commercially available herbicides in a characterisation of resistance. Their properties (including Herbicide Resistance Action Committee Grouping (HRAC)) and MOA are described and their chemical structures illustrated in Figure 1.2.

1.3.1. Isoproturon

Isoproturon (IPU) is a member of the urea chemical family (HRAC Group C_2), which inhibits photosynthesis at Photosystem II (PSII). It is a post-em, selective, systemic herbicide primarily absorbed by the roots and to a lesser extent by foliage. Maximum

activity is achieved from root absorption which is determined primarily by the absorptive capacity of the soil and its moisture content, which in turn affects the persistence and movement of the herbicide within the soil (Orson, 1991). Herbicide injury has been shown to increase if the plant has adequate moisture, as IPU is more active in wet soil. Overhead watering rather than sub-irrigation also increases injury. IPU efficacy and uptake is affected by soils with an organic matter (OM) content greater than 10%. The phytotoxicity of this herbicide increases with light intensity and temperature (Blair, 1986; Orson, 1991).

IPU is a lipophilic molecule, translocated to its site of action in the leaf chloroplasts by means of the xylem and phloem. Foliage uptake of the herbicide and its subsequent translocation is largely acropetal, accumulating in the leaf tips with minimal export from the treated leaf. Selectivity is based on the ability of the species to metabolise the herbicide, as rate of uptake and movement within the plant contributes only minimally (Orson, 1991). Treatment by IPU initially results in rapid cessation of plant growth followed by developing chlorosis and subsequently necrosis.

1.3.2. Clodinafop-propargyl and Fenoxaprop-P-ethyl

Fenoxaprop-P-ethyl (FE) and clodinafop-propargyl (CP) are members of the AOPP family of graminicides (HRAC Group A) and are both post-em foliar-acting herbicides, which affect meristemic tissue. FE is a selective herbicide with contact and systemic action, whereas CP is primarily systemic in nature. These herbicides are mostly absorbed by the foliage of the plant with translocation to the roots acropetally and basipetally (Roberts, Hutson, Lee, Nicholls and Plimmer, 1998) and are relatively unaffected by the parameters of soil type, absorptive capacity, OM, moisture content and light. However, greater toxicity occurs if they are applied during times of high relative humidity, which aids absorption and translocation (AgrEvo UK, 1998; Dekker, 1999).

Table 1.4. Herbicides approved for the control of black-grass grouped according to mode of action.

Grp	Mode of Action	Chemical Family	Active Ingredient	Crops
A	Inhibition of acetyl CoA carboxylase (ACCase)	Aryloxyphenoxypropionates ('fops')	Clodinafop-propargyl	Wheat
			Diclofop-methyl	C & B
			Fenoxaprop-P-ethyl	Wheat
			Fluazifop-P-butyl	B
			Propaquizafop	B
			Quizalofop-P-ethyl	B
		Cyclohexanediones ('dims')	Cycloxydim	B
			Sethoxydim	B
			Tepraloxydim	B
			Tralkoxydim	C
B	Inhibition of acetolactate synthase (ALS)	Imidazolinones	Imazamethabenzmethyl	C
		Sulfonylureas	Flupyrsulfuron-methyl	Wheat
		Sulfonylaminocarbonyl-Triazolinone	Propoxycarbazone-sodium	Wheat
C_1	Inhibition of photosynthesis at photosystem II	Triazines	Atrazine	Maize
			Cyanazine	C & B
			Simazine	C & B
			Terbutryn	C
		Triazinones	Metribuzin	Pots
C_2	Inhibition of photosynthesis at photosystem II	Ureas	Chlorotoluron	C
			Isproturon	C
			Methabenzthiazuron	C
			Metoxuron	C
D	Photosystem-1-electron diversion	Bipyridyliums	Paraquat	N
F	Inhibitors of carotenoid biosynthesis	Nicotinanilides	Diflufenican (only in mixtures)	C
G	Inhibition of EPSP synthase	Glycines	Glyphosate	N
H	Inhibition of glutamine synthease	Phospinic acids	Glufosinate-ammonimum	N
K_1	Microtubule assembly inhibition	Dinitroanilines	Pendimethalin	C & B
			Trifluralin	C & B
K_3	Inhibition of cell division	Chloroacetamides	Metazachlor	B
		Carbamates	Carbetamide	B
		Acetamides	Napropamide	OSR
		Benzamides	Propyzamide	B
			Tebutam	B
N	Inhibition of lipid synthesis – not ACCase inhibition	Thiocarbamates	Tri-allate	C & B
		Benzofuran	Ethofumesate	B
Z	Unknown	Arylaminopropionic acid	Flamprop-M-isopropyl	C
		Others	Difenzoquat	C

KEY: Grp – Herbicide Resistance Action Committee Code **Crops** – Crops which herbicides are approved for use in **C** – Cereals, **B** – Broadleaved crops, **N** – Non-crops/non selective (Source: AgrEvo, 1997b; Moss, 1997; Schmidt, 1997; Whitehead, 2001)

These herbicides are lipophilic and are strongly absorbed into the epicuticular waxes of the cuticle, easily crossing the plasmalemma and then slowly desorbing into the plant apoplast before subsequent intracellular metabolism and translocation. Translocation within the plant is limited, but active transport via the phloem and passive movement in the xylem ensures that the portion that does translocate, does so rapidly to the primary lethal action area – the meristems (apical and root) and areas of rapid growth in the plant. Although herbicide toxicity is determined by the amount reaching the target site (TS), in this instance just a few molecules at the active site are highly effective in susceptible species. The majority of herbicide applied in this case stays at the site of application and symptoms are commonly initially observed at this point in the form of leaf chlorosis. Uptake, translocation and metabolism within resistant and susceptible species is similar (Carr, Davies, Cobb and Pallett, 1986; Cobb, 1992; Roberts *et al.*, 1998).

The first symptom of AOPP application is often rapid cessation of growth due to inhibition of acetyl CoA carboxylase (ACCase) activity. De-esterification occurs in leaf cells and the phytotoxic acid accumulates at the apical meristems causing growth inhibition within days. Secondary effects follow over a longer period as leaf chlorosis, which is usually the first visible symptom in young leaves, and eventually necrosis occurs. Plant death usually occurs within 2 to 3 weeks after treatment (Cobb, 1992).

1.3.3. Sethoxydim

Sethoxydim (SETH) is also a post-em ACCase inhibitor (HRAC Group A), but belongs to a different chemical class of graminicides to FE and CP, the CHDs. It is a selective herbicide, predominantly absorbed by the foliage and to a lesser extent by the roots of plants (Tomlin, 2000). This chemical is photochemically very unstable and exhibits rapid degradation in light, water, soils and on plants. It is weakly absorbed to OM, but other features such as pH and cation exchange capacity are less important factors in soil activity

(Roberts *et al.*, 1998).

Sethoxydim has the same MOA and acts in a similar way to AOPPs, but is very labile and produces several metabolites of which many are phytotoxic to meristems. It is taken up rapidly by the foliage and is translocated both acropetally and basipetally within the plant to accumulate in the actively growing meristems. As SETH affects the same TS as AOPPs, it is not surprising that plant injury is similar, but CHDs tend to have slower rates of penetration into the leaves (Cobb, 1992).

1.3.4. Flupyrsulfuron-methyl

Flupyrsulfuron-methyl (FPM) is powerful post-em sulfonylurea herbicide (HRAC Group B) which inhibits acetolactate synthase (ALS) through foliar and root activity. Its selectivity is metabolism based and its primary attribute is that it has very high specific activity and is thus effective at very low dose rates (Teaney, Armstrong, Bentley, Cotterman, Leep, Liang, Powley, Summers, Cranwell, Lichtner and Stichbury, 1995). FPM is primarily affected by water solubility affecting soil sorption and mobility of the chemical, as does OM and other soil properties (Roberts *et al.*, 1998).

Flupyrsulfuron-methyl is active via rapid foliar and root residual activity uptake resulting in subsequent translocation of the active via phloem and xylem to plant growth centres distant from the point of application. Plant cell division and growth are rapidly inhibited. It is also thought that the a.i. may accumulate within intracellular compartments such as the chloroplast, which is the main location of ALS (Roberts *et al.*, 1998). Efficacy of FPM is a result of ALS inhibition coupled with plants' varying ability to metabolise the chemical. Hence, there is a correlation between the rate at which a plant metabolises it and its tolerance or susceptibility. This is demonstrated by susceptible black-grass metabolising FPM more slowly (half-life of 20 h) than more tolerant wheat and wild oat species (half

life ≤ 2 hours) (Koeppe, Barefoot, Cotterman, Zimmerman and Leep, 1998).

Symptoms of treatment are very similar to other ALS inhibitors in that initially there is rapid cessation of growth in susceptible species. It inhibits mitosis and cell elongation due to a lack of the essential amino acids leucine, iso-leucine and valine, which are required for protein synthesis. Chlorosis and necrosis follow. Anthocyanin accumulation is also an associated symptom (Cobb, 1992). The pattern of injury is a function of the site of herbicide uptake; for example, root uptake influences the pattern of translocation and symptoms of treatment observed which are different if the same herbicide was taken up primarily by the foliage. The time taken for symptoms of herbicide treatment to appear and death to occur may vary between species (Dupont, 1998; Roberts *et al.*, 1998).

1.3.5. AC210

AC210 is a new herbicide, which has been recently launched in the UK commercially by BASF plc. It is a combination of old (pendimethalin) and new (flufenacet) chemistry and can be applied both pre- and post-em.

Pendimethalin is a member of the dinitroaniline chemical family (HRAC Group K_1) and is readily absorbed by both the roots and shoots of treated plants. Dinitroanilines are primarily applied pre-em but some are also post-em herbicides. When applied, the unaltered herbicide binds to tubulin and then interferes with cell division and growth (Roberts *et al.*, 1998). Tubulin is a protein, which forms microtubules through polymerisation. Microtubules form spindle fibres which enable chromosomes to separate during cell division. Dinitroanilines prevent the polymerisation of tubulin. Microtubules also orientate cell wall microfibrils and thus the shape of cells. If this is disrupted, the cells do not elongate to their normal shape. Together, these MOA lead to cells containing an abnormal number of chromosomes (Appleby and Valverde, 1989). The resulting effects of

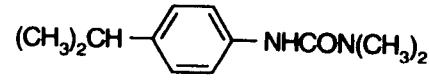
these chemicals lead to disruption of early seedling development with weeds dying soon after germination or following emergence from the soil. Pendimethalin is a residual, selective herbicide, which both inhibits microtubule formation and causes microfibril disorientation. It is strongly absorbed to soil, therefore high OM content is important (Cyanamid, 1997).

Flufenacet (HRAC Group K_3) is an oxyacetamide herbicide whose primary TS is unknown at present, although its MOA is similar to pendimethalin in that it inhibits both cell division and growth. It may inhibit fatty acid metabolism but, as with other oxyacetamides, further research is required for confirmation of the MOA (Deege, Förster, Schmidt, Thielert, Tice, Aagesen, Bloomberg and Santel, 1995; Tomlin, 2000). As a formulation, AC210 is a selective herbicide, which affects meristematic tissue by inhibiting both cell growth and division a few hours after application and results in complete arrest of cell division in both the shoot and root meristematic regions. In susceptible populations, growth is severely affected with the inhibition of both root and leaf meristems, leading to fresh growth being totally suppressed. Elongated tissue may become distorted and the mitotic index is reduced (Deege *et al.,* 1995). Studies have revealed that foliar application leads to little of the compound being translocated basipetally to the root and shoots of the plants. However, treatment via the roots leads to large amounts being translocated acropetally to the shoot. Therefore, it is concluded that flufenacet is translocated mainly through the xylem (Peter Tayler, Cyanamid, Personal Communication, 2000). Adsorption of flufenacet is affected by high OM content and high clay content of soils and may persist in soil for some time. Application rates are dependant upon soil type, region and climatic conditions for reliable control of some grass species (Gajbhiye and Gupta, 2001).

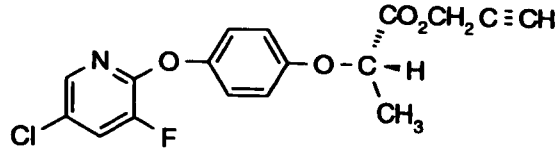
As a combination of the 2 chemicals, ACH210 has broad-spectrum activity against numerous annual grass weeds and recommended applications rates are significantly lower

than current commercial standards. This herbicide is extremely slow acting with no effects visible for up to 3 months, after which control of susceptible populations is good. In addition, herbicidal efficacy of this compound is maximised against very young grass weeds at the 1-2 leaf stage (Deege *et al.*, 1995).

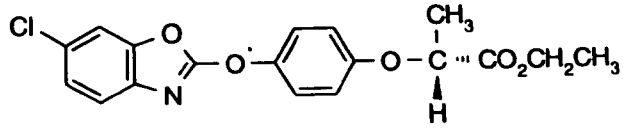
Isoproturon



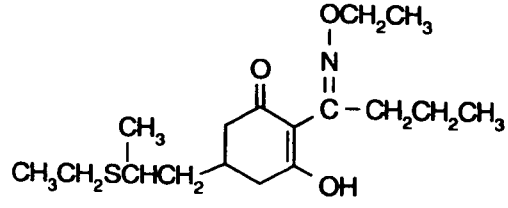
Clodinafop-propargyl



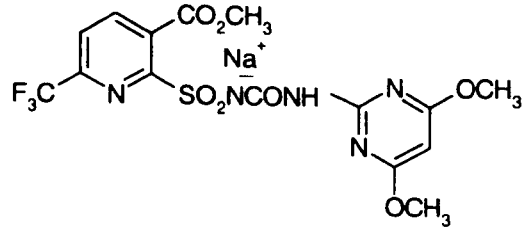
Fenoxaprop-P-ethyl



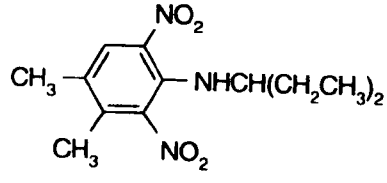
Sethoxydim



Flupyrsulfuron-methyl



Pendimethalin



Flufenacet

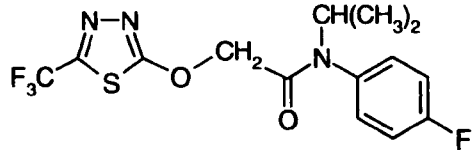


Figure 1.2. Schematic drawings of the chemical structures of the specific graminicides studied in this thesis. (Source: Tomlin, 2000).

1.4. HERBICIDE RESISTANCE IN BLACK-GRASS

Over the last 50 years, the introduction of selective herbicides has revolutionised weed control in the developed world and they are rapidly being adopted in developing countries. In many cropping systems, herbicides are seen as the most reliable and least expensive method of weed control available (Heap, 1997; Powles, Preston, Bryan and Jutsum, 1997). However, despite being a major contributing factor to world crop production, the continuing success of herbicides is threatened by the evolution of herbicide resistance.

Herbicide resistance is defined as the inherited ability of some weed biotypes within a naturally occurring population to survive a herbicide treatment that would, under normal conditions of use, give effective control of that population. Selection of resistant biotypes could eventually result in complete control failures. This inherited ability contrasts with poor activity resulting from incorrect herbicide application or adverse environmental conditions (Heap, 1997; Moss and Clarke, 1995).

1.4.1. Resistance to graminicides

Grass weeds are a major source of competition in the commercial production of most annual, biennial and perennial crops. Thus, the economic success of producing these crops relies upon a grower's ability to control grass weeds in conjunction with other weeds, pests and diseases. Many new graminicides have been developed and commercialised since their introduction in the 1970's and many possess similar MOA. Their effectiveness has meant high usage in cereals. This has resulted in many grass weed populations with natural resistance to herbicides surviving, due to a lack of competition from sensitive populations which are being controlled to a very high level (Graham, 1993). The emergence of graminicide resistance is relatively recent worldwide and has occurred in varying degrees amongst grass species. Worldwide incidence of graminicide resistance in

weeds is now widespread, with resistance and cross resistance to AOPPs, CHDs and dinitroanilines present in important arable weeds. TS cross resistance and multiple resistance in rigid rye-grass (Australia) and black-grass (Europe) present two of the most problematic grass weeds in the world, with few herbicides available unaffected by resistance. Resistance is also confirmed in wild oat species, which is widespread in all major wheat producing regions of the world, with multiple resistant populations appearing recently in North America. Wild oat species are already considered one of the most threatening cereal weeds in the world and are poised to become the worst herbicide resistant weed of wheat (Heap and LeBaron, 2001). The extent and current status of graminicide resistance depends upon the frequency of evolutionary events which confer resistance to the phenotype and absolute size of the population experiencing selection (Mortimer, 1993). Of the 254 resistant weed biotypes worldwide, grass biotypes comprise 40%, indicating that the *Poaceae* family has the greatest propensity to evolve resistance to herbicides and possess more resistant species than any other in the world (Heap, 1999 and 2002). Grass weed species account for all the cases of weed species that have evolved multiple resistance to five or more MOA as illustrated in Table 1.5. The first three account for hundreds of thousands of hectares worldwide and represent the most problematic cases of resistance (Heap and LeBaron, 2001).

Grass weed resistance has been prominent for the last two decades in Europe and Australia. Resistance to graminicides in the USA and Canada is an emerging problem with resistance being found on a small but increasing number of sites. The evolution of multiple resistance in wild oats from Canada is posing a serious threat as there are few herbicides remaining which exert selective control of these populations. In addition, the evolution of glyphosate resistance in rigid rye-grass from Australia, the USA and, most recently, South Africa, as well as glyphosate resistance in goosegrass from Malaysia,

indicates that even with the adoption of glyphosate-resistant crops, resistance strategies will continue to play a vital role in arable farming practices (Heap 1999 and 2001).

Table 1.5. Grass weed members of the top 25 worst herbicide –resistant weeds weighted by propensities in countries, MOA, Sites and Hectares which have evolved multiple resistance to five or more MOAs. (From Heap and LeBaron, 2001)

Species	Common name	No. Countries	No. MOA	No. Sites	No. Hectares	No. Cropping Regimes	Position
Lolium rigidum	Rigid Rye-grass	16	8	7,000	836,400	6	1
Avena fatua	Wild oat	16	6	22,100	2,941,200	4	2
Echinochloa crus-galli	Barnyardgrass	15	6	1,200	817,600	4	6
Eleusine indica	Goosegrass	5	5	6,300	20,100	6	7
Poa annua	Annual meadow grass	15	6	1,100	5,200	4	13
Alopecurus myosuroides	Black-grass	13	4	1,900	9,300	3	14

Resistant grass weeds in the UK are a significant and increasing problem for farmers. To date three resistant grass weeds (all annuals) have been identified and their incidence is on a constant increase: black-grass, wild oat species and Italian rye-grass. Particular interest has been paid to resistance in the UK in recent years with the introduction of AOPP and CHD herbicides and the rate at which resistance to them has evolved (Moss, Horswell, Froud-Williams and Ndoping, 1993; Moss *et al.,* 1999). Several surveys have been carried out in the UK to establish the extent of graminicide resistance. Black-grass resistance is widespread and discussed in further detail in this section. Resistant Italian rye-grass is less frequent. By 1999, resistance to diclofop-methyl had been detected on 30 farms distributed over 12 counties in England. Cross resistance to herbicides with the same or different MOA often occurs in this weed. Herbicide resistant wild oat species have only recently been detected in the UK, with resistance detected in both *A. fatua* (common wild oats) and *A. sterilis* spp. *ludoviciana* (winter wild oats). By 1999, resistance to FE had

been detected on 65 farms distributed over 19 counties in England with cross resistance often occurring (Moss *et al.*, 1999).

To ensure uniformity in the way the world responded to the threat of herbicide resistance, the Herbicide Resistance Action Committee (HRAC) was established in 1989 under the patronage of the International Group of National Associations of Agrochemical Manufacturers. Consisting of technical representatives from herbicide manufacturers HRAC has the objective of managing and delaying herbicide resistance in weeds (Jutsum and Shaner, 1992). To enforce the objective of HRAC and help combat resistant grass weeds within the UK, the UK Weed Resistance Action Group (WRAG) was also set up in 1989 comprising of representatives from British Agrochemical Association member companies and other organisations involved in herbicide resistance research. This group serves to give advice and promote new ideas and technology with respect to the prevention and management of herbicide resistant grass weeds. It is predicted that the occurrence of graminicide resistance and cross resistance is of major significance both to current and future weed control programmes whether it be worldwide or within the UK (Mortimer, 1993).

The cost of preventing herbicide resistance is not as much as having to deal with its presence. An HRAC project has evaluated the cost of preventing black-grass resistance at approximately £90 ha^{-1} and once resistance is obtained this will rise by an additional £90 ha^{-1} (Orson and Harris, 1997; Orson, 1999).

1.4.2. Prevention, management and control of herbicide resistant grass weeds.

Grass weed resistance calls for appropriate measures to prevent build up of resistant populations and for careful management and control of existing resistant populations. The most current recommendations in the UK have been compiled by WRAG, edited by Moss

(1997), providing comprehensive guidelines for the prevention and management of herbicide resistant grass weeds. At present, prevention or management of these weeds requires a long-term, planned strategy incorporating cultural, cropping and chemical programmes similar to those detailed for black-grass control in Tables 1.3. and 1.4.

1.4.3. Incidence and distribution of herbicide resistant black-grass.

Herbicide resistant black-grass has been reported in several countries around the world. Resistance to triazines was reported in Israel in 1982 (Yaacoby *et al.*, 1986) which was soon followed by documentation of chlorotoluron (CTU) resistant biotypes which are now widespread throughout Europe (Niemann and Pestemer, 1984; Moss and Cussans, 1985; De Prado, Menendez, Tena, Caseley and Taberner, 1991; Letouzé, Gasquez, Vaccara, Orlando, Leterrier, Roy and Bouvard-Derieux, 1997). Resistance to AOPPs, CHDs and dinitroanilines has also been documented throughout Europe (Moss, 1990b; Menendez, De Prado, Jorrin and Taberner, 1993; Moss and Clarke, 1995) and in Australia, Canada, and the USA (Mortimer, 1993).

With respect to the number of weed species resistant to herbicides in the UK, (23), black-grass is the most common with four entries of resistance to ureas and amides (1982), ACCase inhibitors (1982), ALS inhibitors (1984) and dinitroanilines (1987) (Heap, 2002). Black-grass resistance in the UK is widespread, with resistance to one or more herbicides confirmed on 746 farms (Moss *et al.*, 1999). This can be broken down into resistance to different chemical classes:

- Resistance to substituted ureas (CTU or IPU) has been detected on 127 farms.
- Resistance to AOPP herbicides (FE or CP) has been detected on 691 farms.

However, some farms fall into both groups, hence the total figure of 746. This figure is likely to have passed 750 at time of writing. Five counties in particular have very high incidence of resistance: Lincolnshire (117 farms), Essex (103), Oxfordshire (92),

Cambridgeshire (75) and Suffolk (58).

The UK has approximately 50,000 farms growing cereals, of which 20,000 are estimated to have a black-grass problem. However, the total number of farms with confirmed resistance only represents 3.7% of this 20,000. The majority of farms in the UK with a black-grass problem (>90%) have not had any samples tested for resistance. However, the 3.7% should only be treated as a minimum figure of the proportion of farms affected, not, as an indication of the proportion of farms with resistance. To date the most resistant population in the UK is found at Peldon in Essex, which exhibits resistance to numerous (but not all) herbicides across many different herbicide classes, including AOPPs, carbamates, dinitroanilines, phenylureas and triazines (Kemp, Moss and Thomas, 1990; Hall, Tardif and Powles, 1994a; Moss *et al.*, 1999). Black-grass is now resistant to many of the herbicides currently approved for its control (Moss, 1987b; Moss and Clarke, 1992).

1.4.4. Development of resistance in black-grass

An early report by Harper (1956) suggested that the development of herbicide resistance is an evolutionary process and proposed characteristics and conditions, which were most likely to influence its occurrence and the speed at which evolution may occur. Black-grass as an arable weed possesses many of these characteristics:

- a high reproductive capacity, meaning populations can build up rapidly, enabling selection to occur;
- absence of a large dormant seedbank to act as a 'buffer' to population changes which reduces dilution by old seeds;
- allogamous reproduction;
- genetic and phenotypic plasticity;
- absence of crop rotations. Black-grass is commonly associated with cereal monoculture.

- intensive use of a single herbicide type leading to resistance problems particularly with the adoption of cereal monoculture (Moss and Cussans, 1985; Moss and Clarke, 1995).

Resistance may be suspected if a herbicide fails to control populations effectively, but herbicide use is not the sole pre-requisite for the development of resistance. LeBaron and Gressel (1982) suggest that there is no evidence that herbicides are the direct cause of resistance, nor that they have had any mutation effect on the natural susceptible population. Moss and Clarke (1992) who report no clear association between the occurrence of resistance and intensity of use support this. The development of resistance in black-grass therefore, may be due to a number of influential factors.

1.4.2.1. Genetic variation and herbicide resistance in black-grass: The material for evolution is the natural inherent genetic variation in populations and is initially dependent upon how much is present (Putwain, 1982; Moss and Rubin, 1993; Powles *et al.*, 1997). However, it is difficult to predict the initial frequency of a resistant genotype in any weed population due to inherent differences between species, locality and type of resistance. Studies of the genetic structure of black-grass indicate that there is a high level of genetic polymorphism in the morphological characteristics of plants and very low genetic differentiation within populations (Chauvel and Gasquez, 1994; Cavan, Bliss and Moss, 1998). However, Chauvel and Gasquez (1994) also indicate low genetic differentiation among populations of wide geographical origin due to similar levels of intrapopulation variability. This is contradicted by Cavan *et al.*, (1998) who found larger variations and larger interpopulation genetic differences. However, Chauvel and Gasquez, (1994) do acknowledge that the lack of genetic variation between distant populations is difficult to explain, as the population distribution of black-grass is discontinuous and the weed is mainly prevalent in winter cereals. The fact that black-grass exhibits at least two resistance mechanisms, suggests that populations will show differing levels of genetic

variation for resistance (Willis, Mortimer, Putwain and Moss, 1997). The identical frequencies found between studied black-grass populations can only be explained by pollen dispersal (close populations) allowing constant gene exchanges, or by seeds (distant populations) with crops. Further evidence suggests that there is no difference between resistant and susceptible populations in any genetic parameter after herbicide selection (Chauvel and Gasquez, 1994, Cavan *et al.,* 1998).

1.4.2.2. Inheritance of resistance: Genetically, there are two ways in which resistance traits may arise in weed populations: Unselected weed populations may have a major resistance-conferring gene, or genes, present at low frequencies or they may arise as a result of mutation and gene flow from the wild type or elsewhere. Subsequent selection with herbicides acts to change a weed population, which is initially susceptible to herbicide treatment (Maxwell and Mortimer, 1994; Diggle and Neve, 2001). Most cases of resistance found in the field are due to the action of a single gene with a high degree of dominance (Jasieniuk, Brûlé-Babel and Morrison, 1996). Alternatively, continuous (polygenic) variation in herbicide response may exist. Recurrent selection with herbicides will result in a progressive increase in resistance from generation to generation, with changes in gene frequency at many loci conferring resistance (Maxwell and Mortimer, 1994).

Due to the varying levels of resistance to CTU in black- grass, it has been suggested that its mode of inheritance is nuclear and polygenic and that resistance is partial. The trait for resistance could be transmitted by pollen (Moss and Clarke, 1992; 1995). Inheritance studies of the resistant biotype Peldon, indicate that at least two nuclear genes with additive action are responsible for resistance to CTU (Chauvel, 1991). This polygenically inherited mechanism may also explain the partial resistance exhibited by the UK Lincs E1 population, which has been subject to less intensive phenylurea treatment than that at

Peldon. However, evidence has been shown for a single CTU resistance mechanism in Peldon due to increased activity of cytochrome P450 enzymes (P450s) (Hall, Moss and Powles, 1995). Inheritance of resistance due to TS insensitivity in black-grass has been identified as monogenic and resistance is absolute (Moss and Clarke, 1995).

1.4.2.3. Herbicide selection pressure: Selection pressure relates to the survival rate of resistant and susceptible individuals after herbicide application (Gressel, 1991). Herbicides are generally applied at rates which give 90-99% mortality of susceptible weed species and thus exert intense selection pressure (Diggle and Neve, 2001). If genetic variation for resistance is present due to mutation or gene flow, even at low frequencies, repeated herbicide applications will result in a rapid increase in the frequency of resistant individuals until they dominate the population (Jasieniuk *et al.*, 1996). Applications of residual herbicides will impose high selection pressure as successive flushes of germinating weeds are exposed to the herbicide. As a consequence there will be a high proportion of resistant individuals in the surviving weed population. Foliar acting, contact herbicides with no residual activity can also exert high selection pressure with a shorter duration period, if there are repeated applications whenever weeds emerge (Moss and Rubin, 1993). Several factors that are likely to influence selection pressure as shown in Table 1.6.

Herbicides are intensive selective agents and where genetic variability for herbicide response exists in weed populations, the evolution of resistance is the inevitable consequence (Diggle and Neve, 2001).

Table 1.6. Factors influencing herbicide selection pressure

(Source: Putwain, 1982; Moss and Rubin, 1993; Shaner, 1995)

Factor	Explanation
Intensity of use	Relates to herbicide effectiveness. Repeated applications of certain herbicides would impose high selection pressure.
Duration of selection	Period of time during which selection acts i.e. a residual herbicide will exert pressure for longer than a contact herbicide.
Efficiency of the resistance mechanisms	The more effective they are, the greater the selection in favour of resistant individuals.
Specificity of the herbicide in terms of mode of action	The more specific the mode of action, the greater the chance of inactivation by a single gene mutation.
Pattern of weed emergence	Selection would only occur when weeds germinate during periods of herbicidal activity.
Effectiveness of any non-chemical control methods	Herbicide selection pressure is reduced by cultural control practices thus reducing weed populations.

1.4.2.4. Plant fitness and seedbanks in the soil: There is a perception that there is a phenomenal and intrinsic feature of the herbicide trait whereby resistant weeds lose another key factor when they gain resistance, resulting in 'reduced fitness' (Moss and Rubin, 1993). Studies between susceptible and resistance populations of black-grass have shown that there is no difference in plant fitness or development (Chauvel and Gasquez 1991, 1994; Sharples, Sanders and Cobb, 1993, Sharples and Cobb, 1996; Sharples, Hull and Cobb, 1997).

Rate of increase and the persistence of the buried seed bank in the soil control the response to increased selection. Due to the short dormancy and limited lifespan of black-grass seeds in the soil, the importance of seedbanks with respect to black-grass resistance is considered relatively minor (Naylor, 1972c; Moss, 1985).

1.4.2.5. Mating systems in black-grass: There is an assumption that mating between plants will be completely random which assumes self-incompatibility and total outcrossing

between individuals within a population (Gressel and Segel, 1978; Gressel and Segel, 1990; Mortimer, Ulf-Hansen and Putwain, 1991). However, this is rare as most weed species exhibit at least low levels of selfing and a number are highly self-fertilising (Brown and Burdon, 1987). A comparison between expected and observed levels of heterozygosity in black-grass indicate that there is no departure from random mating. This could be due either to the seed bank containing seeds from different generations or to spatial structure at the field scale (Chauvel and Gasquez, 1994).

1.4.2.6. Gene flow in black-grass: Gene flow from resistant populations through pollen or seed movement can provide a source of resistant alleles in previously susceptible populations in adjacent fields which in turn can lead to evolution of resistance (Jasieniuk *et al.*, 1996). It is not clear whether resistant patches of black-grass in fields is due to independent mutations or if resistance gene flow by the movement of pollen or seeds is present (Cavan and Moss, 1997; Cavan *et al.*, 1998). Studies suggest that genes are generally exchanged at random within a field. This may be a reflection of the crop rotation adopted and farm practices distributing seed around the field, therefore the field boundary could correspond to the limit of the effective population (Chauvel and Gasquez, 1994). Experiments utilising polymerase chain reaction (PCR) indicate that close genetic relationships between resistant and susceptible black-grass plants have evolved by mutation as opposed to movement of pollen or seed from the same or nearby fields, but the possiblity cannot be ruled out (Cavan and Moss, 1997; Cavan *et al.*, 1998).

1.4.2.7. Rate of resistance development: The existence of genetic variability, its mode of inheritance, selection pressure, herbicide(s) used together with agro-ecological, biological and grower factors determine the rate at which resistance will develop and progress and mean that it varies greatly (Mortimer, 1993; Tardif and Powles, 1993). If the rate of selection is slow, there is time to devise and implement appropriate strategies to minimise

its impact. However, in a situation where there is a rapid build up of resistance, the problem may evolve and develop so quickly that absolute failure of herbicides may occur within a few years (Moss and Clarke, 1995). Resistance can develop in as little as three years from the first application of the herbicide(s) (Tardif and Powles, 1993). The reasons for rate of resistance development are unclear and it is difficult to establish due to the number of possible factors involved.

Several mathematical models have been developed which take into account the ecological, genetic and physiological processes associated with the evolution and spread of resistance. However, it is impossible to predict accurately the occurrence of resistance and its spread, although research is making progress in this area and existing models can help to direct this. These models can be used to consider management options, which may delay the onset of resistance, but their limitations in not being able to predict specific outcomes must be recognised. When there is greater understanding of each factor affecting resistance development, more accurate predictions of rates of resistance development will become possible.

Ten years after the introduction of phenylurea herbicides, black-grass populations resistant to CTU were identified in the UK. Partial resistance was first found in Faringdon, Oxfordshire in 1982 and more pronounced resistance at Peldon, Essex in 1984 (Moss and Cussans, 1985). Moss and Clarke (1995) reported that black-grass was resistant to commonly used urea herbicides, but it did not appear to be a rapidly growing phenomenon. There is no evidence that resistance has spread from a common source and levels of resistance vary quantitatively between populations. It is not clear whether this is due to differences in the frequency of the genes in different populations, to differences in the expression of monooxygenases or if resistance mechanisms vary (Moss and Cussans, 1991). Resistance to CTU has developed at a relatively slow rate (Moss, 1992) and it has

been recognised that the rate of development of resistance to other herbicides might differ to that of substituted urea herbicides.

It was concluded that the incidence of 'marginal' resistance to AOPP and CHD herbicides was of great concern due to the potential for rapid development of resistance (Moss and Clarke, 1992; Mills and Ryan, 1995). Many black-grass biotypes exhibit resistance to diclofop-methyl and FE, but the level and extent of resistance to other AOPPs varies considerably (Moss, 1992). Highly resistant biotypes remain relatively rare, but the evolution of partial resistance is a common occurrence (Mortimer, 1993). Studies have demonstrated that resistance to fenoxaprop can evolve within three generations and is more likely to develop where high ratios of herbicides are used and at a faster rate than CTU. However, resistance to CP occurs at a slower rate than to FE, and the differences between these chemicals from the same class is not yet fully understood. Therefore it is said that the rate of development of resistance is highly variable depending upon the frequency of resistant biotypes present in the population and the herbicides used (Mills and Ryan, 1995; Moss and Clarke, 1995).

The underlying reasons for the rate of resistance development to different herbicides in black-grass are unclear. It is suggested that it may be linked to differences in the rate of metabolism between herbicides, but gene flow via pollen and seed should also be considered (Jasieniuk *et al.*, 1996).

1.5. TYPES OF RESISTANCE

Herbicide resistance in plants relates to MOA of the particular herbicide. The increasing incidence of herbicide resistance has led to two main types of resistance being identified,

cross and multiple resistance. There are no accepted definitions of cross and multiple resistance (Rubin, 1991; Moss and Rubin, 1993).

1.5.1. Cross resistance. Cross resistance is a genetically endowed mechanism whereby individual plants or a weed population are resistant to herbicides from different chemical classes. It can be conferred either by a single gene or by quantitative inheritance influencing a single mechanism (Hall *et al.*, 1994a; Powles and Preston, 1995). The degree of resistance varies amongst populations due to differing levels of genetic variation and it also varies between herbicides, but is not related directly to chemical groups or MOA (Kemp *et al.*, 1990; Moss and Clarke, 1995; Willis *et al.*, 1997). It is complex in nature, partly due to multiple mechanisms of resistance and the suggestion of intra and inter population phenotypic variations in patterns of cross resistance to several chemicals (Moss and Clarke, 1995; Willis *et al.*, 1997). There are two broad cross resistance categories: target site (TS) cross resistance and non-TS cross resistance. Target site cross resistance occurs when there is a change at the biochemical site of action in the plant of one herbicide which also confers resistance to herbicides belonging to a different chemical class, that inhibit the same site of action. However, resistance may not be conferred to all herbicide classes with a similar MOA or all herbicides within a particular herbicide class. Non-TS cross resistance occurs as resistance to herbicide classes due to a mechanism(s) other than resistance enzyme target sites (Powles and Preston, 1995).

Black-grass and rigid rye-grass were the first two grass species to exhibit cross resistance to a range of chemical classes and it is only of relatively recent occurrence (Hall *et al.*, 1994b). Cross resistance in black-grass is widespread and studies have revealed that resistance to phenylurea herbicides in black-grass is associated with cross resistance to a range of other herbicides from differing chemical groups with differing MOA. Cross resistance in black-grass was first documented in populations resistant to CTU and IPU

which were found to be cross resistant to diclofop-methyl and pendimethalin in the UK (Moss, 1990b) and also in Spain (De Prado *et al.*, 1991). Further studies have shown that populations of black-grass exhibit noticeable intra- and inter-population phenotypic variation in patterns of cross resistance to CTU, FE and tralkoxydim (Willis *et al.*, 1997). Chlorotoluron resistant Peldon has been shown to exhibit cross resistance to over 23 herbicides from a range of chemically unrelated classes (Moss and Clarke, 1992). As discussed, the degree of resistance varies between herbicides, but is not directly related to chemical groups or MOA e.g. CTU resistant black-grass from Peldon, Essex has been shown to be cross resistant to pendimethalin but not to trifluralin, although both herbicides are dinitroanilines (Moss, 1987b; Moss, 1990b; Kemp *et al.*, 1990; Moss and Clarke, 1995). Cross resistance in black-grass is an extensive problem for both UK farmers and agrochemical manufacturers, as there are few chemical alternatives. Moss and Clarke (1995) suggest that the extent of cross resistance in the UK cannot be characterised on individual fields as it may change within a short time period, thus herbicides should be considered separately.

1.5.2. Multiple resistance

Although cross resistance is a problem, weeds which exhibit multiple resistance now are and in the future will be, more difficult to control. Multiple resistance is the expression of more than one resistance mechanism within individual plants (Hall *et al.*, 1994b) and can lead to simultaneous resistance to few or many herbicides with different MOA. Multiple resistance was relatively unknown until the 1990s and is regarded as complex and difficult to control. In its simplest form, individual plants or populations possess two or more different resistance mechanisms conferring resistance to a single herbicide or herbicide class. More complex forms are those where two or more resistance mechanisms have been sequentially or simultaneously selected by different herbicides, and resistance has been conferred to the herbicide classes to which they have been exposed (Powles and Preston,

1995). The mechanisms present in a population can include one or more mechanisms, which confer cross resistance (Hall *et al.*, 1994a). Multiple resistance has evolved in some weed populations, most notably black-grass in Europe (Kemp *et al.*, 1990; Moss, 1990b; Mendenez *et al.*, 1993) and numerous populations of rigid rye-grass in Australia (Hall *et al.*, 1994b; Tardif , Preston and Powles, 1996).

Although a problem in Europe, multiple resistance in black-grass not widespread. The UK biotype Lincs E1 exhibits widespread non-TS cross resistance as a result of increased metabolism to several herbicide classes following selection with CTU. It also exhibits resistant ACCase individuals following selection with an ACCase herbicide and is highly resistant to FE (Hall *et al.*, 1995; 1997). The resistant Peldon biotype is different to Lincs E1 suggesting that multiple mechanisms may or may not occur in different populations. As black-grass populations possess an allogamous mating system which under continuous selection pressure favours mechanistic enrichment, some populations may possess both TS and EM resistance mechanisms at varying frequencies within populations (Hall *et al.*, 1994b). Multiple resistance is problematic for farmers particularly in situations where a number of resistance mechanisms, involving both TS and non-TS resistance mechanisms are present as resistance occurs concurrently to many or all herbicide options available enforcing the adoption of integrated weed management strategies.

1.6. RESISTANCE MECHANISMS

Herbicide resistance can be conferred by several distinct mechanisms and herbicides are capable of selecting all that are available. Resistance mechanisms are the method by which resistant plants overcome the effects of exposure to herbicide treatment. The ability of plants to survive exposure to herbicides may be the result of biochemical/physiological, morphological alterations or phenological changes (Moss and Rubin, 1993). However,

most cases of resistance are the result of either enhanced metabolism (EM) of herbicides to inactive products or insensitivity at the TS. Other potential resistance mechanisms include the reduced uptake of the herbicide or translocation into cells or tissues and sequestration and compartmentation. The basis of resistance in the latter mechanisms is often the result of morphological and physical factors and it is virtually impossible to attribute a single mechanism to the resistance. These mechanisms are all similar to some crop selectivity mechanisms which enable them to survive exposure to herbicides, but the specific mechanisms of herbicide resistance in weeds usually differ substantially from those which are responsible for crop selectivity (LeBaron and McFarland, 1990; Gasquez and Darmency, 1991; Devine and Preston, 2000). The mechanism(s) present within weeds will influence the pattern of resistance, in particular the cross resistance profile and dose response (HRAC, undated).

It is stated that knowledge of the biochemical basis of resistance is required to develop resistance management strategies (Cocker, Moss and Coleman, 1999). However, few detailed studies have been carried out on weed populations. To aid management strategies, information based on a wider range of representative populations is required. Unless a wide range of weed populations are evaluated, the chances of discovering and identifying all the mechanisms, which may be responsible for resistance are reduced. This is of particular importance because recent research on biochemical mechanisms has focused on the suggestion that resistance in some weeds may be conferred by multiple resistance mechanisms at the whole-plant level (Tardif and Powles, 1994). The two most common resistance mechanisms are TS insensitivity and EM, which can occur alone or in combination with each other.

1.6.1. Target site insensitivity

Target site insensitivity is the most common resistance mechanism in herbicide resistant

weeds world-wide and is expressed when a herbicide no longer binds to its normal site of action within the plant, usually due to a structural change at the molecular level (HRAC, no date). Resistance is absolute and does not affect chemically unrelated herbicides. Target site insensitivity is conferred by a mutation of the target site protein. The modified form of the enzyme still functions within the plant but is insensitive to inhibition by herbicides - hence the survival of resistant plants (Moss and Clarke, 1995, Devine and Preston, 2000). Resistance may also be due to over-expression or an increase of the target protein, but this is rare and has only been found in cases of recurrent selection or under laboratory conditions (Devine and Preston, 2000). Studies of the genetics of TS resistance indicate that inheritance of this trait is monogenic involving a single, nuclear gene and that the resistant allele is dominant to the susceptible. In practice, the consequences of this are that TS resistance builds up rapidly if affected herbicides are used repeatedly. No evidence of incomplete dominance has been found (Tardif and Powles, 1993; Moss and Clarke, 1995). The main chemical groups affected by TS resistance and their major sites of herbicide action are shown in Table 1.7. There are now more confirmed cases of ALS inhibitor resistance than to any other herbicide group (Heap, 2001). With respect to the widespread use of selective graminicides and grass weeds, TS resistance is an intensifying problem, particularly affecting the ACCase inhibitors as illustrated in Table 1.7.

Resistance to these herbicides leaves few other chemical options available to farmers and therefore the detection of this mechanism when only a small proportion of plants are affected is essential in order to delay its build up through repeated use of AOPP and CHD herbicides (Moss and Clarke, 1995). The simplicity of the monogenic nature of TS resistance allows it to build up rapidly in contrast to the more complex, gradual development of polygenic EM hence its is more of a problem worldwide (Moss and Clarke, 1995).

Table 1.7. Chemical groups affected by target site resistance, the major sites of herbicide action which they target and weed resistance examples.

(From Devine and Preston, 2000)

Chemical group(s)	Target site	Process inhibited	Weed example(s)
s-Triazines, phenylureas, uracils	Q_B protein	Photosynthetic electron transport	*Brassica rapa* *Portulaca oleracea* *Senecio vulgaris*
Sulfonylureas, imidazolinones, triazolopyrimidines	Acetolactate synthase	Branched-chain amino acid biosynthesis	*Galium spurium* *Lactuca serriola*
Glyphosate	enol-Pyruvylshikimate-3-phosphate synthase	Aromatic amino acid biosynthesis	*Lolium rigidum* *Eleusine indica*
Cyclohexanediones, aryloxyphenoxypropionates	Acetyl-CoA carboxylase	Fatty acid biosynthesis	*Avena fatua* *Avena sterilis* *Lolium rigidum* *Eleusine indica* *Setaria viridis* *Setaria faberi* *Alopecurus myosuroides*
Dintroanilines, carbamates, phosphoric amides	α-,β-Tubulin	Cell division	*Eleusine indica* *Setaria viridis*

TS resistance in black-grass was first documented by Moss and Clarke (1995) and solely affects the AOPP and CHD herbicides. Despite being the most common mechanism worldwide, this mechanism is less common than EM in the UK, but when it occurs, populations are highly resistant and its incidence in the UK is rapidly increasing. Black-grass studies have revealed high levels of resistance to AOPP and CHD herbicides but not to chemically unrelated herbicides such as CTU in biotypes Lincs E1, Oxford S1, Notts A1 and Oxford A11 (Moss and Clarke, 1995).

1.6.2. Enhanced metabolism

This is a broad-spectrum mechanism, whereby a resistant plant degrades a herbicide to non-phytotoxic metabolites faster than a susceptible plant and affects the performance of a wide range of chemically unrelated herbicides (HRAC, undated; Moss and Clarke, 1995).

This mechanism is also the basis of tolerance in many crops (Shimabukuro, 1985; Cole, 1994). Resistance due to EM results in widespread and varied cross resistance to many herbicides as well as in-between populations. Enhanced metabolism resistance is partial, as opposed to absolute, and inheritance of this trait is probably nuclear and polygenic (Chauvel, 1991; Moss and Clarke, 1995).

The development of resistance due to EM is partially attributed to high selection pressure imposed by repeated herbicide applications (Orson and Harris, 1997). Many weed biotypes have evolved resistance to herbicides by possessing the ability to rapidly degrade and/or conjugate the herbicides into less toxic compounds but only two: *Abutilon theophrasti* (velvet leaf) and *Stellaria media* (chickweed) are dicotyledons. The rest are grass weeds found worldwide which are proposed to have evolved resistance due to EM and are shown in Table 1.8.

The two most notable grass weeds exhibiting resistance due to EM, as seen in Table 1.8., are black-grass in Europe and rigid rye-grass in Australia, usually due to enhanced hydroxylation and dealkylation reactions (Kemp *et al.*, 1990; Burnet *et al.*, 1993, Preston and Powles, 1997). Recent research has also identified an enhanced rate of metabolism of FE conferring resistance in two partially resistant UK wild oat populations (Cocker, Coleman, Blair, Clarke and Moss, 2000). The most recent discovery has been the first report world wide of resistance due to EM to diclofop in Italian rye-grass in the UK (Cocker, Northcroft, Coleman and Moss, 2001).

Resistance due to EM in black-grass is endemic in the UK and is the most common type of resistance to date. It is widespread and results in varied cross resistance to many herbicides as well as in-between populations (Moss and Clarke, 1995; Moss, 1997). CTU and IPU are more rapidly metabolised in the resistant population Peldon (oxidation then

rapid conjugation) compared to susceptible populations (Kemp *et al.*, 1990; Hall *et al.*, 1995; Sharples and Cobb, 1996; Hyde, Hallahan and Bowyer, 1996). Peldon also exhibits more rapid detoxification of diclofop-methyl and FE (Hall *et al.*, 1995; Menendez and De Prado, 1996). The rapid degradation appears to be due to processes associated with cytochrome P450 enzymes (P450s). Cross resistance to other herbicides may be the result of detoxification by similar processes (Kemp *et al.*, 1990; Hall *et al.*, 1995). Some herbicides such as FE are vulnerable to both resistance mechanisms. In particular, studies have shown that both mechanisms are present in some European populations of black-grass which exhibit resistance to FE (Cocker *et al.*, 1999).

Table 1.8. Grass weed species with confirmed resistant populations due to increased metabolism of herbicides. (From Devine and Preston, 2000)

Weed species	Herbicides(s)	Proposed enzymatic system
Alopecurus myosuroides	Chlorotoluron Pendimethalin Diclofop-methyl Fenoxaprop-P-ethyl Propaquizafop Chlorsulfuron	Cytochrome P450 monooxygenase Glutathione *S*-transferase?
Avena sterilis	Diclofop-methyl	Cytochrome P450 monooxygenase
Avena fatua	Triallate	Cytochrome P450 monooxygenase[a]
Digitaria sanguinalis	Fluazifop-P-butyl	Unknown
Echinocloa colona	Propanil	Aryl acylamidase
Echinocloa crus-galli	Propanil	Aryl acylamidase
Hordeum leporinum	Fluazifop-P-butyl	Unknown
Lolium rigidum	Simazine Diclofop-methyl Fluazifop-P-butyl Tralkoxydim Chlorsulfuron Metribuzin Chlorotoluron	Cytochrome P450 monooxygenase Unknown
Phalaris minor	Isoproturon	Cytochrome P450 monooxygenase

[a] resistance due to decreased activation of herbicide.

1.6.3. Other mechanisms

The presence of additional resistance mechanisms in plants is widely recognised. Moss and Clarke (1995) discovered that neither EM nor TS resistance could explain the degree of plant resistance to FE and fluazifop shown by one particular population of black-grass at the whole-plant level and thus the presence of another resistance mechanism was likely. Despite the evidence for P450s being involved in detoxification of black-grass herbicides, not all populations exhibit the same pattern of resistance as the resistant Peldon population, hence a single detoxification mechanism cannot be assumed (Kemp *et al.*, 1990; Moss and Cussans, 1991, Hall, Moss and Powles, 1993, Sharples *et al.*, 1997). The molecular basis of resistance remains to be established, as although EM due to P450s is implicated in resistant Peldon elevated glutathione *S*-transferase (GST) activity has also been observed in Peldon plants. Resistant black-grass plants have been shown to exhibit approximately twice the GST activity of susceptible plants and it is postulated that high GST activity may play a role in herbicide resistance in black-grass (Cummins, Moss, Cole and Edwards, 1997a; Reade, Hull and Cobb, 1997).

The presence of unidentified resistance mechanisms in black-grass is supported by the documentation of a CTU resistant population from Tiptree, Essex found to possess a unique pattern of resistance. No evidence of cross resistance to diclofop or pendimethalin was observed, suggesting either that a number of oxidases may operate selectively in herbicide detoxification or that additional resistance mechanisms may operate in black-grass (Kemp *et al.*, 1990; Reade *et al.*, 1997). Another example is black-grass resistance to FE, which was selected for in a susceptible population after four generations and the mechanism of resistance as yet remains unidentified. The population exhibits cross resistance to flamprop but not to quizalofop, tralkoxydim, CTU or chlorsulfuron. The inheritance of this resistance appears to be nuclear and involves a single gene (Chauvel, Gasquez, Doucey and Perreau, 1992; Reade *et al.*, 1997). Additional studies concerning

FE have revealed that a single resistance mechanism alone does not explain resistance in some European black-grass populations and that additional, as yet uncharacterised, mechanisms must also be present (Cocker *et al.*, 1999).

1.6.4. Prevention, management and control of herbicide resistant black-grass.

Herbicide resistance in black-grass is an increasing problem which requires appropriate measures to prevent the build up of resistant populations as well as careful management and control of existing resistant populations. The current cultural and chemical recommendations are those detailed in Tables 1.3. and 1.4. It is possible that in the future genetically modified crops tolerant to glufosinate-ammonium will be introduced. This chemical has been shown to control AOPP resistant black-grass and may offer an alternative control option (Read, Palmer and Howard, 1997). However, at present, prevention or management of resistant black-grass requires a long-term, planned strategy.

1.7. TESTING FOR RESISTANCE

Herbicide failure does not necessarily indicate herbicide resistance. Investigations of the herbicide application, rate applied, weed type and growth stage, environmental conditions and agronomic practices through field observation should initially be carried out to rule out all possible reasons for herbicide failure. If resistance is still suspected, then consideration of historical information relating to the area should be taken into account, which may point to factors leading to the development of resistance as described by HRAC (undated). If all potential factors for herbicide failure have been eliminated, resistance should be suspected and growers are advised to collect a seed sample or a whole plant for a resistance confirmation test. An economically effective resistance strategy relies on the diagnosis of resistance as a first step, before resistant weeds become established as major components in local weed flora. This requires efficient and effective screening tests. Interpretation of

results is also critical with respect to developing appropriate management strategies (Truelove and Hensley, 1982; Beckie, Heap, Smeda and Hall, 2000).

There are several resistance tests available to growers: laboratory, greenhouse or field techniques, mainly tools available through agronomists, agrochemical manufacturers or government research agencies. Some detect resistance regardless of the mechanism present while others are specific to individual mechanisms. In order to achieve efficient, effective screening, the essential attributes of a diagnostic test are rapidity, accuracy, ability to predict field performance, independently conductible, ready availability and inexpensiveness! (Moss, 1995). However, currently available tests are time-consuming and many do not provide information for the current growing season (Reade and Cobb, 2002). The key factor of resistance tests is the degree of insensitivity, as small differences exhibited in tests may have significant effects on herbicide efficacy in the field and therefore must be interpreted carefully. However, where resistance is absolute, the detection and interpretation of these effects can be difficult. Differences in the resistance status of individual plants within a population must also be considered as they can also influence result interpretation. This is because resistance may be due either to a quantitative increase in the level of resistance of all individual plants within a population or to an increase in the proportion of the very resistant types (Moss, 1995). A comprehensive review about testing for herbicide resistance in weeds has recently been published by Beckie *et al.*, (2000).

1.7.1. Field experimentation

This can be carried out on a suspected resistant population in the same year as herbicide applications are seen to fail. The advantage is that the experiment can be sited in the problem area and is simple to carry out. Information is collected during the same growing season and results can provide recommendations for subsequent crops. Alternatives to this

method are setting the experiment in the subsequent crop, which allows more flexibility and choice in terms of herbicides and application timings. Limitations to this kind of experimentation are that studies set up in the same year are usually carried out late in the growing season, and therefore herbicides will not be applied at appropriate growth stages. Consequently results must be treated with caution. In addition, unless crop damage is disregarded, the choice of herbicides will be limited to those that can be used in that crop. Care must be taken when using repeated applications and doses higher than the recommended rate as they may be illegal unless an experimental permit has been awarded, which may demand crop destruction on conclusion of the study. Field experimentation can provide growers with information which can be put into immediate use. However downfalls include the fact that there is no susceptible standard population for results to be compared against. In addition, it is difficult to assess whether reduced herbicide efficacy is due to resistance or to other unrelated factors, particularly if partial resistance is suspected and if soil-acting herbicides are used whose activity is influenced by environmental conditions (Moss, 1995).

1.7.2. Whole plant studies/pot tests

Until recently, this was the most widely used test for resistance. It is conducted either in a glasshouse or controlled environment chambers. Seed samples are collected from weed populations where resistance is suspected and are grown in pots of soil or nutrient medium alongside standard reference populations. Plants are typically sprayed at GS12/13 with herbicides at one dose rate or, more commonly, a range of doses to calculate a dose response curve. Assessments are carried out 3-4 weeks post treatment, by visual evaluation of mortality or vigour, or by measuring foliage fresh or dry weight. Resistance values are attributed to each weed sample as appropriate. The inclusion of standard reference populations allows this test to be carried out at any time, in any location, and the use of a range of doses means that the degree of resistance can be quantified (Moss and

Clarke, 1992; Heap, 1994; Moss, 1995). This test is simple and effective as it goes some way towards mimicking field applications and can detect resistance regardless of the mechanisms present. However, unless resistance is absolute, it is difficult to predict the likely impact of resistance upon field performance of the herbicides tested. The limitations of this test are the requirement of seeds which have to be collected which may have innate dormancy, the time taken to obtain results (which may not be in time for the following growing season) and the fact that it demands high labour and glasshouse requirements (Moss, 1995; Beckie *et al.*, 2000). Despite these drawbacks, many feel that this test is the most appropriate single test for resistance as the herbicide applications and activity mimic field conditions. Many therefore consider pot studies a good validating test to carry out alongside some of the newer commercially available tests.

In 1999, a resistance test was commercially marketed for testing grass weed survivors of herbicide treatment in the field which is now known as the 'Syngenta Quick Test'. Plants are sampled from the field and posted to the relevant organisation, who take cuttings from the plants, which are subsequently transplanted into pots. The cuttings are allowed to produce new leaves, which are then treated with herbicides. Results are similar to those of plants grown from seed. Advantages of this test are that it has a wide sampling window, as cuttings are suitable from plants that are starting to tiller through to early flowering and it avoids the problem of seed dormancy. Results can be provided within 4 weeks, allowing in-season testing of weed survivors and thus potential to adopt alternative control measures (Boutsalis, 2001).

1.7.3. Petri-dish bioassays

Resistance to a range of herbicides has been successfully evaluated by use of Petri-dish bioassays. In the majority of tests, seeds are geminated on filter paper or agar in the presence of a herbicide and approximately 1-3 weeks post treatment, a growth parameter

such as shoot or root length is assessed (Moss, 1995). Many tests have been carried out this way to determine resistance to ACCase inhibitors, dinitroanilines and triazines (Moss, 1990b) in *Setaria viridis* (green foxtail) (Beckie, Friesen, Nawolsky and Morrison, 1990), rigid rye-grass (Heap and Knight, 1986; Gill, 1990), wild oats (Murray, Friesen, Beauliea and Morrison, 1996) and *Sorghum halepense* (Johnsongrass) (Smeda, Barrentine, Snipes and Rippee, 1997).

A new Petri-dish test marketed commercially is the Rothamsted Rapid Resistance Test which detects resistance to herbicides in black-grass, wild oat species and Italian rye-grass by germinating seeds with specific concentrations of herbicides in comparison to standard reference populations. Shoot length is recorded two weeks after treatment, which provides a measure of the degree of resistance. Advantages of this test are that it gives an indication of resistance mechanisms present and is reasonably rapid as it can provide results by mid-September for seeds collected in July. However, it is geared as an agronomist's tool or for an organisation with laboratory facilities. It also requires skilled labour to conduct what is a simple test with a highly detailed protocol (Moss, 1999).

Two other seedling bioassays have recently been developed for rapid screening of resistance specifically to AOPP herbicides in black-grass, rigid rye-grass and Little seed canary grass based on measuring the difference in coleoptile length (Letouzé and Gasquez, 1999) or shoot and root lengths (Tal, Kotoula-Syka and Rubin, 2000) of resistant and susceptible seedlings grown in herbicide acid solutions. Advantages of these tests are that results can be obtained in 6 or 7 days. They determine the frequency of resistant individuals within a population and discriminate between AOPP resistant populations. The tests are designed to be portable and do not require expensive or specialist equipment. Their main limitations are that acid forms of herbicides are not readily available, that seed dormancy must be overcome and that some seeds do not germinate well in Petri dishes

(Moss, 1995; Letouzé and Gasquez, 1999; Tal *et al.*, 2000). Both tests are being developed for use with other grass weeds.

Petri dish tests have the advantage over pot tests in that they take a lot less time, do not require the same labour and space requirements and are inexpensive. It is possible to screen large numbers of samples with this method. However, limitations include seed collection and dormancy reducing the potential advantage of rapid evaluation of fresh seed samples. In addition, they are not applicable to all forms of resistance and therefore interpretation of results is critical, particularly as herbicide application does not mimic the field, which affects the method and speed of uptake of the herbicides. Single assessments of these tests are not relevant so results must be correlated and compared to whole plant responses. Ideally, petri dish assays should be validated with pot tests using soil (Moss, 1995; Beckie *et al.*, 2000).

1.7.4. *In vitro* assays

In vitro assays are also used to determine herbicide resistance, be it by measuring photosynthetic competence, fluorescence, amount of pigment or protein or activity of a herbicide's target enzyme. The assay used is dependent upon the herbicide's site of action, but limitations include the possibility of false, negative results e.g. triazine resistant *Abutilon theophrasti* Medik. (velvetleaf) gave the same enhanced fluorescence as the susceptible biotype but resistance was due to EM as opposed to TS modification (Gronwald, 1994). Chlorophyll fluorescence is a well-established technique used for studying photosynthetic activity and identification of triazine resistant weeds (van Oorschot, 1991; Rubin, 1992). Fluorescence induction was developed to study the MOA and resistance of photosynthetic inhibitors, especially triazines, at molecular level, using changes in fluorescence signals to give an indirect measure of photosynthetic activity. A dark-adapted leaf, pre-treated with herbicide is illuminated and emitted fluorescence is

measured. Differences in the signal indicate the photosynthetic capacity of the leaves and thus give an indication of their resistance status. However, resistance due to EM is difficult to detect as the degree of resistance is smaller, but when used for determination of quantitative resistance in plants, fluorescence has shown promising results with respect to this type of resistance (van Oorschot and van Leeuwen, 1992). Fluorescence tests correlate to conventional pot tests, but are only really suitable for use with photosynthetic inhibiting herbicides and can be labour intensive (Moss, 1995).

Richter and Powles (1993) demonstrated the use of pollen in the detection of resistance to ACCase and ALS inhibitors in rigid rye-grass and suggested that the technique could form the basis of a rapid resistance test for TS based resistance mechanisms. Letouzé and Gasquez (2000) have developed a pollen test to detect ACCase target-site resistance within black-grass populations at the flowering stage. This assay is based upon the germination of pollen in an agar medium supplemented with ACCase inhibitors whereby the percentage germination rate is measured. The advantage of this bioassay is that it can provide results in two hours and it provides reliable detection of TS resistant plants. In comparison to *in vivo* assays, which are discussed below, pollen tests are simpler as pollen can be readily obtained and no specialist equipment is required. However, it does require skilled labour to produce the agar medium and handle herbicides. This test would provide information detailing which herbicides would still exert satisfactory control of a resistant population and would allow herbicide strategies to be devised for the next growing season by avoiding seed dormancy problems. This test is currently being extended for use in resistant wild oat and rye-grass species (Letouzé and Gasquez, 2000).

1.7.5. *In vivo* assays

Traditionally, assays such as those which detect ACCase and ALS are thought too complex and time consuming to be considered as rapid screening tests, and only provide

information about resistance conferred by TS insensitivity. Therefore resistance conferred by other mechanisms and multiple resistance may go under-estimated or even undetected (Beckie *et al.*, 2000). However, this area has provoked research interest, whereby instead of measuring the activity of a herbicide's TS enzyme, the abundance and activity of enzymes such as GSTs, which catalyse the metabolism of several herbicides with different sites of action, is measured and may form the basis for a rapid screening test for detection of EM resistance (Reade, Belfield and Cobb, 1999; Reade and Cobb, 2002). One particular test has been validated in black-grass with clear observations that assessment of GST activity (by colorimetry) or abundance by enzyme-linked immunosorbent assay (ELISA) may be correlated with resistance due to EM (Reade and Cobb, 2002). At present this technique is laboratory-based working on plants sampled from the field. Results are obtained within 3 days and have been proven to be valid on plants from GS11. Although not currently commercialised, further research is being carried out to develop this test for potential use in the field by agronomists or farmers to avoid enzyme deterioration through handling and transport and to provide results within one day i.e. prior to post-em herbicide application. Preliminary studies of other grass weeds which occur in the UK have indicated that this test may be of use in other species (Reade and Cobb, 2002).

A test of this nature would provide growers with information to make informed decisions while a range of control options are still available. Few assays which require plant material sampled from the field have succeeded, as plants are usually too mature in growth stage once resistance is suspected, and enzymes are labile and deteriorate rapidly once harvested. In addition, field sampled plants will contain a proportion of both resistant and susceptible individuals, therefore homogenised samples may produce a value between reference resistant and susceptible biotypes. Enzyme assays require skilled labour, can be time consuming and more often than not require sophisticated equipment (Beckie *et al.*, 2000). However, in their defence, concurrence between the relative resistance of biotypes

at enzyme and whole-plant level has been observed (Devine and Eberlein, 1997).

1.7.6. Novel techniques

The above techniques and tests are all effective in detecting herbicide resistance, but there is a niche market for a rapid resistance test providing immediate results conducted on either seeds or whole plants sampled directly from the field, as demonstrated by Reade and Cobb (2002). Routine testing for herbicide resistance has become a popular area of research as resistance has such an impact on farm profits and herbicide usage. A new generation of commercial resistance tests has become available in the last few years which confirm resistance in less time and require less expertise. Some of these are mentioned above and many are based on the techniques already described. However, research is ongoing and in particular immunological techniques (ELISA) and DNA analysis techniques have become popular with respect to developing a fast, in-field resistance test.

One such area of research is the use of PCR to amplify genomic DNA fragments and sequence them into resistant and susceptible lines for the detection of ACCase inhibitor resistance in grass weeds. Research is being carried out to see if it may be developed as a diagnostic tool to be used alongside traditional methods (Sinclair, Greenwood, Marshall, Moss, Walter, Mortimer and Putwain, 1999). Assays which could diagnose the specific resistance mechanism present would give quicker, more accurate resistance confirmation, but their precision in itself may be a constraint where multiple resistance mechanisms are present (Moss, 1995). This area of research will remain a priority for many years, as the early detection of resistance is so vital to an effective resistance strategy.

1.7.7. Classification of resistance

In Europe a classification system was devised for the interpretation of results from glasshouse screening tests for CTU resistance in black-grass (Clarke and Moss, 1989).

The * rating system distinguished different degrees of resistance based on a comparison with the percentage reduction in foliage fresh weight values of three standard reference populations (Rothamsted, Faringdon and Peldon) which were included in every resistance test. Samples were classified by a star rating of 1* to 5* or S (susceptible). Only samples given 2* or more were acknowledged as resistant and the higher the rating, the greater the degree of resistance. This system was revised by Clarke, Blair and Moss (1994) using 2 standard reference populations (Rothamsted and Peldon), which was suitable for CTU and FE.

As discussed, the interpretation of the results of resistance tests is critical and the conclusion of a "ring test" was that, disregarding how screening assays are conducted, the basis on which resistance is assigned should be fixed. Many organisations/companies who conduct resistance testing do not interpret results in the same ways when designating degree of resistance, which leads to confusion in the information provided to farmers and the scientific community. Some use the * rating system, whilst other use a simpler version based on visual scores (Moss *et al.*, 1999). With increasing resistance in grass weeds it was recognised that a system was required which is:

- applicable to other species e.g. wild oat and rye-grass species;
- applicable to a wider range of herbicides;
- less dependent upon resistant standards which may only be applicable to a narrow range of herbicides and which may be difficult to maintain in the long term.

A new system was proposed by the UK WRAG, which although based on the * rating system only requires the inclusion of 1 susceptible standard population and this has been adopted by all centres in the UK screening for resistance. The principal reason for adopting this system was to standardise the approach for all grass weeds at different testing centres and thus overcome the problem of provision of standard reference populations.

This system is applicable for wild oat and rye-grass species as well as black-grass, and has all the advantages of its predecessors, but with a reduction in the number of categories and utilising the same susceptible standard for all herbicides (Moss *et al.*, 1999). Results of screening tests for all grass weeds will be disclosed for each sample and herbicide used, using the following categories:

S = Susceptible;

R? = Early indications that resistance may be developing, possibly reducing herbicide performance (previously 1*);

RR = Resistance confirmed, probably reducing herbicide performance (combining previous 2* and 3*);

RRR = Resistance confirmed, highly likely to reduce herbicide performance (combining 4* and 5*).

These categories replace the existing * rating system and are calculated using an agreed procedure to ensure results' interpretation is standardised. The only vital pre-requisite of the system is that control of the susceptible standard is greater than 80%. The system is applicable to the standard pot test as well as newer tests such as the Rothamsted Rapid Resistance Test and the Syngenta Quick Test. There is a risk element incorporated in that the higher the degree of resistance, the greater the risk of herbicide failure (Moss *et al.*, 1999). Protocols, interpretation of results and standardisation of information release has also been adopted in Canada and America following guidelines proposed by the Weed Resistance Working Group of the Expert Committee on Weeds in Canada and the Herbicide Resistant Plants Committee of the Weed Science Society of America (Beckie *et al.*, 2000).

1.8. PLANT DEFENCE AGAINST STRESS

Plants are sedentary in nature and rely on biochemical detoxification mechanisms to defend themselves from stress, chemical and oxidative. However, there is great variation within plant species with respect to the ability to metabolise substances, often the metabolic basis for herbicide selectivity. These mechanisms are both constitutive and inducible, with the ability to offer protection from stress.

1.8.1. Chemical stress

Several non-enzymatic and enzymatic mechanisms act together to detoxify herbicides in plants. Pesticide metabolism and detoxification occurs in 3 stages. Phase 1 - oxidation, hydroxylation or dealkylation, catalysed by P450s, enhances the usually lipophilic pesticide in both reactivity and polarity and modifies it so it is less phytotoxic. Phase 2 - conjugation with natural plant constituents e.g. an amino acid, glutathione (GSH) (catalysed by GSTs) or a sugar moiety, which renders the pesticide inert and water-soluble. Phase 3 – compartmentation of foreign compounds into the vacuole or cellular matrix. Not all examples of metabolism follow this path and many of the enzymes involved have yet to be isolated and characterised (Cole, 1994; Coleman, Blake-Kalff, and Emyr Davies, 1997). Herbicide selectivity is complex but the most important thing for a plant resistant to herbicides is the ability to detoxify the chemical rapidly, in contrast to a susceptible plant which does not possess this ability (Hathway, 1989; Lamoureux, Shimabukuro and Frear, 1991).

1.8.1.1. Phase 1 – Primary metabolism: The cytochrome P450 enzymes

P450s are considered to be the most important enzyme system for Phase 1 metabolism of herbicides as they are able to utilise herbicides as substrates (Frear, 1995; Barrett, 1995, 2000). The most common reactions to herbicides are hydroxylations and demethylations

resulting in a hydroxylated or dealkylated metabolite of the parent herbicide (Cole, 1994). Some P450 functions and the particular P450 performing the function will exist in all plants. However, research indicates that the majority of P450s in a specific plant are involved in secondary metabolism, which is species specific and contributes to the differences in herbicide metabolism and selectivity exhibited by crop species and their weeds (Barrett, 2000). The activities of P450s can be constitutive or induced by stress and they play roles in both detoxification and defence with the diversity of P450s being related to the roles they take in secondary metabolism (Hendry, 1986).

Herbicide metabolism due to P450s was first recorded in cotton detoxifying diuron (Frear, Swanson and Tanaka, 1969). However, the next documentation of this phenomenon was not until 1990 and the metabolism of bentazon in maize microsomes (McFadden, Gronwald and Eberlein, 1990). Since then the metabolism of numerous herbicides by P450s has been identified in maize, rice, sorghum and wheat, but it is suggested that there are many other herbicides, metabolised by P450s, yet to be identified. It has been found that the same herbicide can be metabolised by P450s that are members of different families, implying that multiple P450s in a specific plant or across a species are capable of metabolising a particular herbicide. In some cases P450s only partially detoxify the herbicide and a second reaction such as conjugation is required for complete detoxification. In other scenarios, although P450s detoxification occurs, the conjugation of the herbicide with GSH catalysed by GSTs is considered the selective detoxification reaction e.g. metolachlor in maize (Barrett, 2000).

Unfortunately, the development of a herbicide with no danger to the crop where there is total differential detoxification between crop and weeds is impossible. The role of P450s in herbicide resistant weed species is well-documented (Jones, 1991; Hyde, Cheriton, Hallahan and Bowyer, 1993; Werck-Reichhart, 1995). Several populations of grass weeds

have evolved resistance due to enhanced P450 metabolism of herbicides, including black-grass (Kemp and Caseley, 1987; Kemp *et al.*, 1990; Hall *et al.*, 1995; Menendez and De Prado, 1996). In the majority of cases, this is due to enhanced hydroxylation reactions, although in black-grass there is also evidence of increased dealkylation reactions (Kemp *et al.*, 1990; Burnet *et al.*, 1993; Hall *et al.*, 1995; Preston and Powles, 1997). Within resistant weed species, increased activities of specific P450s have been identified with different P450s metabolising different herbicides, for example in a population of rigid rye-grass resistant to a number of herbicides where several P450 inhibitors inhibited the metabolism of 5 different herbicides (Christopher *et al.*, 1994; Preston, Tardif, Christopher and Powles, 1996; Preston and Powles, 1997). However, one P450 is also capable of metabolising several herbicide substrates be it from the same chemical family or different. There is no evidence to suggest that similar herbicide selection will enhance the activity of the same P450 in all species, as demonstrated by CTU resistant rigid rye -grass (due to ring-hydroxylation reactions) demonstrating cross resistance to IPU (Devine and Preston, 2000). A feature of resistance due to EM by P450s is cross resistance, which many resistant weed species demonstrate to many herbicides from differing chemical classes with the multiple detoxification activities present due to different P450 activities (Preston, Tardif, Christopher and Powles, 1996). Examples include that of CTU resistant black-grass, which is cross resistant to AOPP and dinitroaniline herbicides (Kemp *et al.*, 1990; Menendez and De Prado, 1996; Hall *et al.*, 1997). There is no evidence that a single P450 is totally responsible for the EM of different herbicides. The activities of many P450 are also exhibited in susceptible biotypes of all these species, but at much lower levels (Barrett, 2000). Herbicide resistance could also arise due to a mutation in a P450 following gene duplication whereby an amino acid change in a P450 protein sequence could alter its substrate specificity. Additional evidence suggests that resistant populations constitutively express higher levels of P450s than susceptible biotypes for which evidence has been documented in a CTU resistant rigid rye-grass population (Preston and Powles,

1997). The importance of P450s and herbicide metabolism and selectivity has increased with the introduction of new herbicide chemistry e.g. sulfonylureas (Owen, 1987; Barrett, 1995). However, further research into enhanced detoxification by P450s is required (Barrett, 2000; Devine and Preston, 2000).

1.8.1.2. Phase II – Secondary metabolism

Once in Phase II, the xenobiotic or Phase I metabolite is deactivated by conjugation to an endogenous hydrophilic molecule. This is often the final phase of herbicide metabolism and can take many forms. Substrates that are conjugated to amino acids, malonic acid and sugars become water soluble conjugates destined for tranport into the vacuole but the reaction is reversible. In contrast, conjugation to GSH is irreversible. Phase II reactions are catalysed by glucosyl-, malonyl and GSTs producing non-toxic or considerably less toxic products and are an important part of the detoxification process in terms of plant protection (Coleman *et al.*, 1997). For the purpose of this thesis, this section will concentrate on GSH conjugation. For a comprehensive and detailed review of secondary metabolism of agrochemicals in plants, the reader is referred to Cole and Edwards (2000). Conjugation of herbicides with reduced GSH forming conjugates of reduced toxicity is an important and irreversible mode of detoxification in crops and weeds (Shimabukuro, Lamoureux and Frear, 1978; Cole, Cummins, Hatton, Dixon and Edwards, 1997; Cole and Edwards, 2000). A range of agrochemicals are susceptible to GSH metabolism. Xenobiotics containing electrophilic sites are of particular danger to plants, as they exert cytotoxic or genotoxic effects by binding with nucleophilic sites, and different electrophilic agents react better with different nucleophilic sites. This conjugation occurs by the nucleophilic attack of GSH on xenobiotics with a suitable electrophilic centre. Glutathione has many roles in cellular metabolism and acts both as a reducing agent and protectant of cells from oxidative stresses as an antioxidant and is the most abundant form of organic sulphur in plants (Foyer, Lelandais and Kunert, 1994a, Hell, 1997). It is also a

nucleophile, which protects against toxicity via the reactions of Phase II. Glutathione also functions as an intracellular signalling agent in response to the external environment (Rennenberg and Brunold, 1994; Sánchez-Fernández, Fricker, Corben, White, Sheard, Leaver, Van Montegue, Inzé and May, 1997). It can also undergo conjugation, be it spontaneous or catalysed by GSTs to a range of xenobiotic electrophiles both hard and soft. Conjugation with soft electrophiles can be spontaneous (with enhancement from GSTs if required) (Leavitt and Penner, 1979), whereas conjugation with hard electrophiles requires GST catalysation (Lamoureux and Rusness, 1986; Coleman *et al.*, 1997). Many examples of GSH conjugation exist but recently contrasting evidence of herbicide metabolism and detoxification of an AOPP herbicide (FE) through non-enzymatic conjugation with GSH and cysteine in grass species has been observed (Tal, Romano, Stephenson, Schwan and Hall, 1993; Tal, Hall and Stephenson, 1995).

The role of GSH conjugation in herbicide tolerance was first established by Shimabukuro, Frear, Swanson and Walsh, (1971) with respect to maize and atrazine. To date, herbicides metabolised in this way include members of the AOPPs, chloroacetanilides, diphenylethers, thiocarbamate sulphoxides, triazines and sulfonylureas (Jablonkai and Hatzios, 1991; Cole, 1994, Edwards, 1995; Cole and Edwards, 2000). In the majority of cases, the parent herbicide is sufficiently reactive to undergo direct GSH conjugation, but in a few cases bioactivation is required e.g. metribuzin. Agrochemical conjugation with GSH catalysed by GSTs occurs by nucleophilic displacement at various molecular sites or by nucleophilic addition, for example tridiphane, but this is a rare occurrence (Dixon, Cummins, Cole and Edwards, 1998, Edwards and Dixon, 2000). Both reactions are illustrated in Figure 1.3. Conjugation to GSH takes place in the cytosol, but if left to accumulate its products are potentially harmful. For example, the conjugates may block the activity of GSTs (product inhibition) which would lead to a build up of electrophiles which could not be conjugated, or conjugates may encounter cytosolic enzymes which

cytoplasm and their compartmentation is of critical importance for plant survival and effective detoxification (Coleman *et al*, 1997).

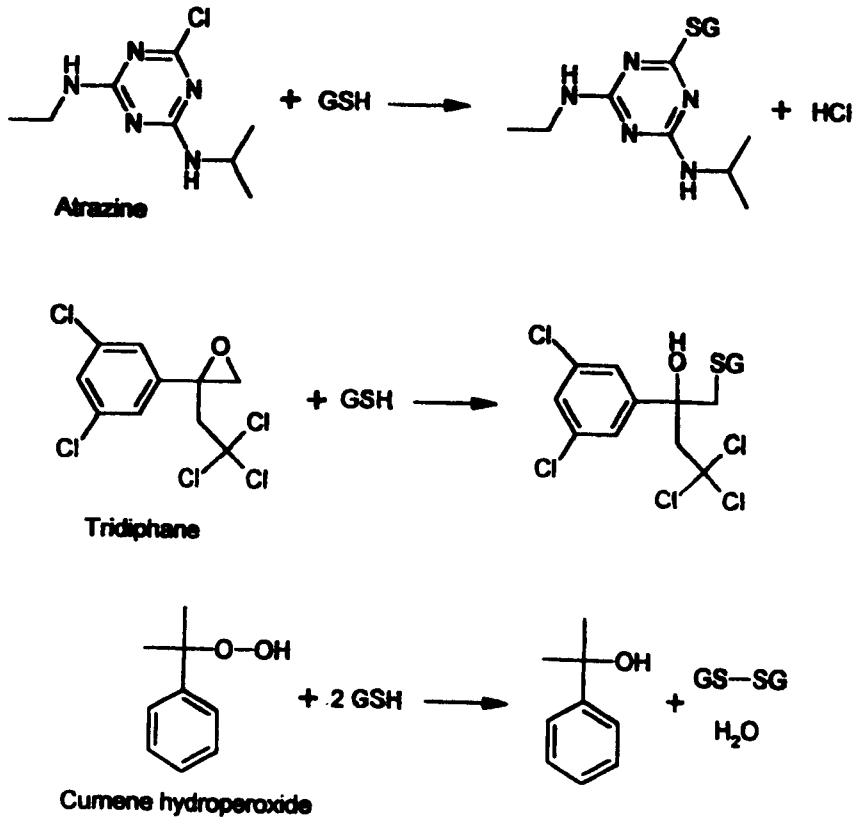


Figure 1.3. GST-mediated detoxification reactions with xenobiotics. GST activity in crops and weeds catalysing the detoxification of atrazine and tridiphane by nucleophilic displacement and addition reactions with GSH respectively.

(Source: Edwards and Dixon, 2000).

1.8.1.3. Phase III – Compartmentation

The products of Phase II (inactive, water-soluble conjugates) and herbicides are excreted from the cytosol to the vacuole and apoplast by membrane located transport proteins, which initiate Phase III, the compartmentation and processing part of the detoxification process (Coupland, 1991; Coleman *et al.*, 1997). Transport of GSH conjugates requires them to cross the tonoplast or plasma membrane. The pH of the cytoplasm is approximately 7.4, thus the conjugates meet a negative charge and hence membrane transport by electroneutral (nonionic) diffusion or even simple diffusion due to the energy barrier is not possible. This is overcome by the presence of an ATP-dependent transporter, which recognises GSH conjugates as substrates (Martinoia, Grill, Tommasini, Kreuz and Amrhein, 1993; Coleman *et al.*, 1997). On arrival, the conjugates are acted upon by a carboxypeptidase (Wolf, Dietz and Schroder, 1996). A second peptide bond cleavage occurs and a *S*-cysteinyl derivative is formed, which may be returned to the cytoplasm or subjected to further transformations (Cole and Edwards, 2000). This mechanism allows plants to sequester functionally diverse yet structurally similar molecules into the vacuole. However, the vacuole is an active environment and metabolites can potentially re-enter the cytoplasm, so this mechanism is not necessarily one of confined disposal (Cole, 1994).

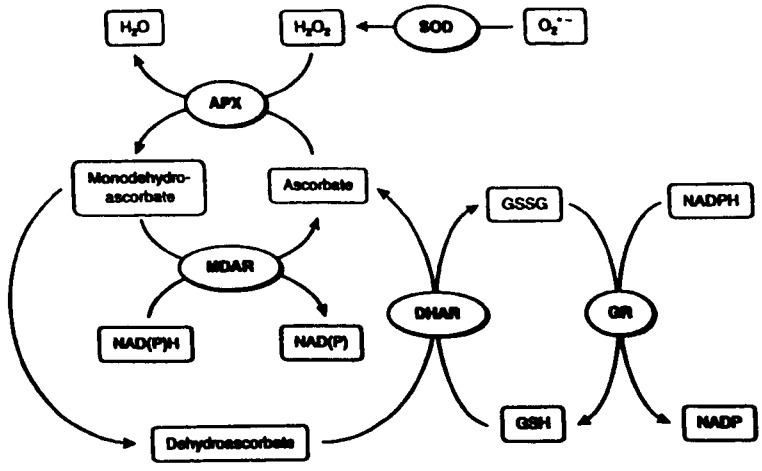
1.8.2. Oxidative stress

Under aerobic conditions, many different situations induce oxidative stress whereby elevated levels of active oxygen species (AOS) e.g. singlet oxygen, hydroxyl radicals, superoxide anions and hydrogen peroxide are formed in plant cells. These situations include environmental stress e.g. ozone, UV light, salinity and cellular senescence, as well as herbicide application and pathogen attack (Foyer and Mullineaux, 1994; Inzé and van Montagu, 1995; Alscher, Donahue and Cramer, 1997). To deal with AOS, higher plants have developed complex protection mechanisms, more commonly known as the antioxidant defence system. Plants can adapt their antioxidant concentrations in response

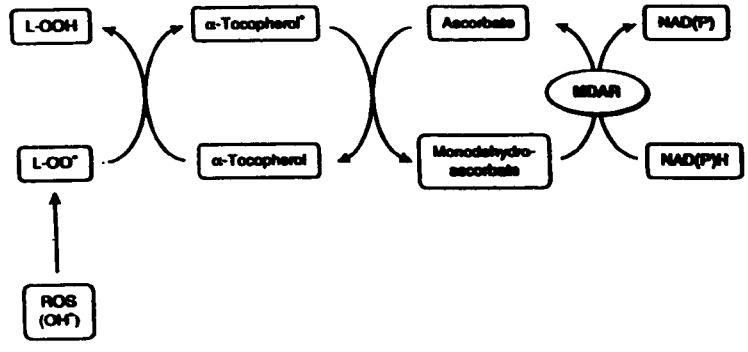
to environmental stress and herbicide treatment. The main pathway of AOS detoxification is presented in Figure 1.4a.

Superoxide dismutase (SOD) scavenges superoxide anions which arise from photoreduction of oxygen from photosystem I (Mehler reaction) or from the chemical reduction of oxygen, resulting in hydrogen peroxide (H_2O_2) and oxygen (O_2) by the dismutation of two molecules of $O_2^{\cdot -}$. Hydrogen peroxide is a toxic metabolite itself and is therefore scavenged by the ascorbate-glutathione cycle (the Halliwell – Asada pathway) in the chloroplast (Foyer and Halliwell, 1976; Nakano and Asada, 1980). The reduction of H_2O_2 to water is catalysed by ascorbate peroxidase (APX) utilising ascorbate (AsA) as an electron donor. Ascorbate is oxidised by APX proceeding in a one electron step forming the monodehydroascorbate radical (Nakano and Asada, 1981). This then either spontaneously disproportionates to give AsA and dehydroascorbate (DasA), or is reduced to AsA by NADPH which is catalysed by monodehydroascorbate reductase (MDAR) (Hossain, Nakano and Asada, 1984; Winkler, Orselli and Rex, 1994). Dehydroascorbate reductase (DHAR) using GSH as a reductant is significant in the reduction of dehydroascorbate to AsA. Glutathione disulfide (GSSG) is then NADPH-dependently reduced by glutathione reductase (GR) (Halliwell and Foyer, 1978). As a result, photosynthesis via NADPH directly provides the ascorbate – glutathione cycle with its reductants. This pathway acts mainly in the chloroplast, but isoforms of the enzymes involved have also been detected in the cytosol (Jiménez, Hernández, del Río and Sevilla, 1997).

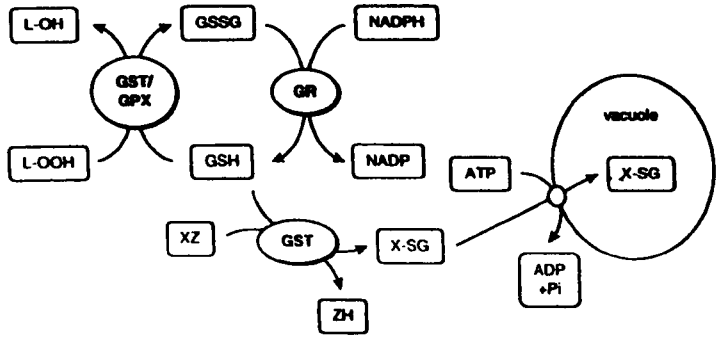
Numerous low molecular weight antioxidants act as free radical scavengers. By reducing polyunsaturated fatty acid peroxyl radicals of membrane lipids, the lipophilic α-tocopherol interrupts radical chain reactions during lipid peroxidation and is recycled via AsA (Figure 1.4b), the hydrophilic counterpart of α-tocopherol. Ascorbate and GSH are major



(a) Ascorbate-glutathione pathway (Halliwell-Asada pathway)



(b) Ascorbate/α-tocopherol pathway



(c) Glutathione pathway

Key: **APX** – ascorbate peroxidase; **DHAR** – dehydroascorbate reductase; **GR** – glutathione reductase; **GPX** – glutathione peroxidase activity; **GST** – glutathione *S*-transferase; **MDAR** – monodehydroascorbate reductase; **L-OO** – peroxyl radical; **L-OOH** – lipid hydroperoxide; ***α-tocopherol*** – chromanoxyl radical; **XZ** – xenobiotic or toxic metabolite (note that hydroxyalkenals can also be subject to glutathione conjugation); **Z** – nucleophile moiety displaced in the GST-catalysed reaction; **X-SG** – glutathione conjugate.

Figure 1.4. Pathways of antioxidative defence and glutathione-mediated detoxification in higher plants (From Knörzer and Böger, 1999).

radical scavengers in the aqueous compartments of plant cells (Foyer and Halliwell, 1976; Alscher, 1989; Luwe, Takahama and Heber, 1993). Glutathione acts as protection for free sulfhydryl groups of proteins against oxidation by free radicals (Kunert and Foyer, 1993). In addition, antioxidant enzymes such as glutathione peroxidase (GPX) and GSTs may also provide protection against AOS. Glutathione peroxidases scavenge lipid peroxides during lipid peroxidation (Eshdat, Holland, Faltin and Ben-Hayim, 1997). A similar role has been proposed for GSTs, which normally detoxify xenobiotics and toxic metabolites but they are known to have multiple functions, which give rise to a GSH pathway (Figure 1.4c).

1.8.3. Glutathione *S*-transferases

Glutathione *S*-transferases (GSTs EC 2.5.1.18) are a large group of related proteins constitutive in all eukaryotes which detoxify electrophilic xenobiotics by catalysing their conjugation with GSH (Edwards, 1995). Plant GSTs were first discovered catalysing the conjugation of atrazine in maize and other related species in 1970 (Frear and Swanson, 1970). This demonstrated the importance of these enzymes and led to the discovery that many herbicides are detoxified by GST catalysed GSH conjugation, with the suggestion that they catalyse the majority of conjugation of agrochemicals *in vivo* (Lamoureux *et al.*, 1991; Cole and Edwards, 2000). Interest in GSTs was initiated because of their involvement in herbicide detoxification and is now well established. In contrast, the role of GSTs in endogenous metabolism is relatively unknown and largely unexplored but there is great interest in the roles GSTs play in endogenous metabolism, particularly as they are shown to regulate responses to plant development and environmental stress (Mauch and Dudler, 1993; Marrs, 1996; Roxas, Smith, Allen and Allen, 1997; Dixon *et al.*, 1998; Edwards, Dixon and Walbot, 2000). For detailed reviews concerning GSTs and their various roles, the reader is referred to Marrs, (1996) and Edwards and Dixon, (2000).

GSTs are a family of supergenes and related proteins. They are soluble enzymes of

approximately 50 kDa, composed of 2 subunits, each of molecular mass in the range of 20-25 kDa. The site responsible for binding herbicides and other xenobiotics is found in the C-terminal half of the subunit where there is a hydrophobic ligand binding domain. This site varies in structure between GSTs, which accounts for the specificity differences exhibited by different GSTs to xenobiotics. The orientation and binding of the hydrophobic ligand, at the same time as the sulphydryl group of GSH, is activated to its thiolate anion species for *S*-conjugation reactions forms the basis of the catalytic mechanism of GSTs. If GSTs are absent, the thiolate anion will only form under basic conditions. In rare instances, the herbicide may be so electrophilic that spontaneous reaction with GSH at pH 7.0 will occur. However, in the majority of cases, they will only undergo significant rates of conjugation with GSH when catalysed by a GST (Coleman *et al.*, 1997; Edwards, 1997). The active sites of GSTs are found in the cleft between the 2 subunits and they function independently of each other, despite the fact that in herbicide metabolism GSTs are only active as either homodimers or heterodimers (Marrs, 1996; Edwards *et al.*, 2000). GST activity towards herbicides and other xenobiotics is primarily located in the soluble fraction, with a small amount associated with the membrane fraction (Hatton, Cummins, Price, Cole and Edwards, 1998). Compartmentation of GSTs in plant cells is a relatively unknown area (Takahashi, Hasezawa, Kusaba and Nagata, 1995; Edwards and Dixon, 2000).

GSTs are very important in their role as catalysts in herbicide detoxification, but their significance in determining herbicide selectivity is not always recognised. Differences in GST activity are associated with herbicide selectivity and resistance, with both cereals and broadleaf crops exhibiting high levels of GST activity towards herbicides, whereas competing weeds with lower GST activities possess slower metabolism and thus greater herbicide susceptibility than the crop. (Hatton, Cole and Edwards, 1996; Andrews, Skipsey, Townson, Morris, Jepson and Edwards, 1997; Coleman *et al.*, 1997). Many

studies have furthered understanding of GSTs in weeds and have led to the suggestion of a role for GSTs in herbicide tolerance in weeds. Resistance has evolved to encompass numerous herbicides with differing MOA where enhanced herbicide metabolism is the most likely mechanism, with GSTs making an important contribution (Holt, Powles and Holtum, 1993). The first case of resistance due to enhanced GST activity was identified to atrazine and other triazine herbicides in the foliage of a population of velvetleaf as a result of increased herbicide detoxification due to increased expression of GSTs in resistant plants (Gronwald, Andersen and Yee, 1989; Anderson and Gronwald, 1991; Gray, Stoltenberg and Balke, 1995; Gray, Balke and Stoltenberg 1996).

Glutathione *S*-transferases in black-grass resistance is of particular interest with respect to the extent of cross resistance exhibited by this weed to many differing herbicide classes and the fact that EM has been proposed as a resistance mechanism responsible for cross resistance (Edwards and Dixon, 2000). Reade *et al.*, (1997) postulated a role for GSTs in resistance to FE after observing approximately double the GST activity in resistant Peldon black-grass plants than susceptible. This increased activity was constitutive, not requiring induction by herbicides. The major route of detoxification in FE is by GSH conjugation enhanced by GSTs supporting the suggestion that GSTs play a role in black-grass resistance to this widely used graminicide.

Further study of the GST activities in both the Peldon and Lincs E1 populations found that they were significantly higher than in the susceptible Rothamsted population (Cummins *et al.*, 1997a). Antisera were raised to a major GST in wheat and utilised to discover that enhanced GST activities in resistant black-grass are related to the increased expression of an 25 kDa polypeptide and novel 27kDa and 28 kDa polypeptides which are only observed in resistant populations (Cummins, Cole and Edwards, 1999). Reade and Cobb (1999) carried out purification and characterisation studies identifying a constitutive 27.5 kDa

polypeptide in both resistant and susceptible populations and a novel 30 kDa polypeptide in resistant Peldon, supporting the findings of Cummins *et al.*, (1999). The proteins of the 27.5 kDa polypeptide were recognised by the anti- *Ta*GSTU1 serum purified from the black-grass and shown to correspond to an active GST. Antisera were also raised to this GST which indicated that is was immunologically different from the GST-like polypeptides found in the Peldon biotype, but were also notably absent in susceptible populations (Cummins *et al.*, 1997a; Reade and Cobb, 1999). Additional studies were carried out to screen the GST activity towards the artificial substrate 1-chloro-2,4-dinitrobenzene (CDNB) of populations with different resistance traits which indicated that GST activity was associated with black-grass resistance to FE (Reade *et al.*, 1997).

Purification, characterisation and cloning have illustrated the importance of GSTs in herbicide metabolism and selectivity in crops and weeds and in particular, the role that they play in herbicide resistance in weeds. The advantage of this information is that it can be taken to discover and develop new selective graminicides that are detoxifiable by crops, but not weeds, however, that is a long way off, although published research indicating how information about the structure and reactivity of herbicides can be utilised in new agrochemical development. Other potential roles are being discovered for GSTs as agents of selective herbicide bioactivation, particularly in weed-specific herbicide bioactivation. Herbicide metabolism and the role of GSTs remains an active research area, in particular analysis of the factors regulating GST expression and also the modes of GST induction particularly by herbicide safeners (Edwards and Dixon, 2000).

1.9. HERBICIDE RESISTANCE AND THE FUTURE

Herbicide resistance continues to escalate world-wide causing significant yield losses and increasing the cost of food production (Powles *et al.*, 1997). Future trends in weed management are likely to change, with predictions of more changes in agriculture and weed management in the next 20 years than have ever been seen before. Intensive agriculture has been practised in developed countries for decades. Predictably this has led to these countries having serious infestations of herbicide resistant weeds in major crops, in the most productive and fertile areas where herbicides are essential. In the last decade (1990-1999) North America overtook Europe as the continent producing the greatest number of new resistant weed biotypes. In contrast, developing countries in the past have not depended upon herbicide use due to economic restraints and the abundance of cheap labour available. Although they have fewer resistant weed species, they have a dire need for better tools for weed control, including herbicides and simplified rotations. There has been a steady increase in herbicide usage in Central and South America and in Asia (predominantly in wheat and rice growing regions). This has led to increases in the number of new resistant weed species being identified in these parts of the world, particularly in the last two decades. The developing world typically produces one herbicide for one weed problem, has fewer alternatives and is less likely to adopt rotations than the developed world. It is anticipated that there will be a rapid increase in resistance problems in the developing world (Heap, 1999). Patterns of herbicide usage are related to the farming systems practised in different regions, levels of agronomic output and socioeconomic factors. These factors are discussed below and are by no means a definitive list, but can all directly influence the development of resistance and will undoubtedly contribute to what is a spreading problem.

Farmers are likely to be put under growing pressure as the world population increases with

a progressive desire to improve diet quality. Predictions suggest world population will reach 11 billion by 2050. Farmers therefore face the challenge of producing food to sustain acceptable living for all. Industrialisation is already burgeoning in developing countries. This will continue to draw labour from the land and take land out of food production thus exerting pressure on farmers to increase productivity per unit area. Pressure exists to balance the needs of population with conservation and land development (Cobb and Kirkwood, 2000; Glasgow and Reynolds, 2000). Avery (1995) proposed that increasing global population could only be fed by the adoption of intensive agriculture. This undoubtedly will further the advance of resistance. The prediction of increasing pressure on the price of herbicides and competitiveness within the agrochemical industry particularly exhibited in the UK in the last two years will also contribute to the continuing threat of herbicide resistance as farmers buy cheaper chemicals and maintain high herbicide usage.

The general public who consume the produce of farmers demand high quality food at minimal risk. However, public perception in recent years has raised issues such as pesticide residues, food safety and impacts on the environment. This is of particular relevance with the introduction of genetically modified foods and ingredients. Regulations imposed by governments, and public opposition continue to obstruct the mass introduction of existing and new herbicides and genetically enhanced organisms, despite there having been no risk to human health identified from in depth studies (Cobb and Kirkwood 2000; Glasgow and Reynolds, 2000). Despite the incidence of glyphosate resistant weed species, genetically modified crops will be an important tool in the management of existing resistant weeds whilst ensuring the population is fed, but in the long term may run into resistance problems themselves.

Herbicide tolerant crops and their technology have already made a significant impact in

world-wide agriculture, particularly in the developed world in crops such as soybean, maize, cotton and canola. As technology evolves, there will undoubtedly be adoption of other bio-technological enhancements (Glasgow and Reynolds, 2000). New technology, in combination with weed management, will play an important part in the war against resistance. Academia is a major source of information for weed management particularly in the education of growers and industrial personnel. The work that researchers carry out with respect to integrated weed management practices and the monitoring of the development and spread of resistance needs to be continued and disseminated to the farming community in order for resistance to be prevented and controlled (Shaner, 1995).

Unfortunately government policies have a major impact on the implementation of effective herbicide resistance strategies. Restrictions on pesticide usage, elimination of registration of older herbicides, restrictions on reducing cultural practices to prevent soil erosion, these are but a few. Crop subsidy programmes introduced to qualify for insurance and price support may be politically popular, but may also inhibit the implementation of effective strategies. Government agencies such as ADAS in the UK are important research centres which require funding in order to carry their work on the effectiveness of differing weed management strategies to maintain food productivity (Shaner, 1995).

The occurrence of herbicide resistance has led to increased interest in weed ecology with particular attention given to weed populations evolving resistance to herbicides after the continuous use of the same or similar herbicides. Predictions suggest a 'knowledge revolution' where farmers will use site specific technology to fight resistance. Farmers will have to combine and balance cheap, broad spectrum weed control against investment in information gathering, weed mapping, mixing and matching effective products (Glasgow and Reynolds, 2000).

Many governments have introduced programmes of pesticide optimisation but herbicides will remain the most cost effective and reliable form of weed control (Shaner, 1995). Many commercially available herbicides have already come off patent or will do so within the next 5 years, with the inevitable increase of cheap generics. The introduction of new a.i.s has been a major factor in the promotion of proprietary products. However grass weeds are resistant to the majority of MOA available to growers. The industry is long overdue a new MOA. Incidences of resistance will continue to increase until new technology is introduced to prevent or curb it. It may be that the integration of agrochemical companies and biotechnology firms may find the answer to this quest (Glasgow and Reynolds, 2000). The agrochemical industry will also be instrumental in the development of new methods of herbicide application and will play a major role in the implementation of integrated weed management.

Ultimately, the success of anti-resistance strategies and management of existing resistant weed populations lies with growers themselves. If growers do not adopt recommendations then the roles and contributions of the above will be in vain, and resistance will continue to spread ultimately severely compromising food production. However, environmental, technological, economic and social factors together with financial viability for farmers must be taken into account. As a result it is concluded that herbicide resistance is of major significance to both current and future weed control programmes. There is little sign that it will do anything but accelerate in the immediate future.

1.10. AIMS OF THIS INVESTIGATION

Resistance in black-grass is an increasing problem to farmers in the UK, Europe and worldwide. The competitive ability of black-grass and efficiency of resistance mechanisms ensures that resistant populations survive in the field. Research indicates that this is not due to morphological differences in growth between resistant and susceptible plants (Kemp *et al.*, 1990; Sharples *et al.*, 1997) which suggests that resistance mechanisms reside at the biochemical level, presenting a novel research area.

Plants possess a wide array of metabolic systems, both constitutive and inducible in order to protect themselves against stress from biotic and abiotic factors. It is well documented that herbicide treatment induces elevated GST activity in resistant black-grass (Sharples *et al.*, 1995; Cummins *et al*, 1997a; Reade *et al.*, 1997) and herbicide metabolism and detoxification by GSTs is well established (Marrs, 1996). However, the natural roles of GSTs in plants remain largely unexplored and uncharacterised (Edwards and Dixon, 2000). Evidence suggests physiological roles for GSTs as specific GSTs have been shown to accumulate in plants exposed to environmental stress, infection or plant hormones (Marrs, 1996). It may be that natural plant responses to environmental stress through the induction of GSTs may contribute to plants being resistant to subsequent stress e.g. herbicide treatment. There is a requirement to further knowledge with respect to the roles GSTs play in herbicide resistance in black-grass and to investigate the hypothesis that GSTs have natural roles in plant defence against biotic stress which contribute to black-grass plants being resistant to herbicide treatment. Increased knowledge of the metabolic plasticity of black-grass and its ability to acclimate to its surrounding environment and how this contributes to herbicide resistance will be a valuable tool when considering control strategies.

1. The first objective of this study was to characterise black-grass resistance to post-emergence herbicides by comparing the responses of a commercially, uncharacterised susceptible population and the two current resistant and susceptible reference black-grass populations by means of glasshouse based studies. This was novel as no studies characterising black-grass resistance to several of the applied herbicides had previously been carried out before. This study also incorporated a newly introduced resistance classification system, which was innovative with respect to the ratings given to the herbicides used. A further aim was to establish whether the commercial population could be successfully used as a susceptible reference population. Characterisation of resistance in susceptible populations is currently not a widely explored area, yet is critical when interpreting small differences in responses between populations at both whole plant and biochemical levels.

   Further additional characterisation in the form of biochemical laboratory studies was carried out to confirm the role of GSTs in herbicide detoxification and resistance in black-grass, of which the results are presenting in Appendix 1.

2. The second part of this study was an evaluation of herbicide resistance with respect to GST activity in untreated black-grass plants sampled from the field. The aim was to investigate the relationship between GST activity and black-grass growth and development in the field, with the overall aim of testing GST activity as a marker for predicted herbicide efficacy. In-field sampling was carried out over a period of two years in order to determine GST activity in differing UK black-grass populations. Both growth assessments and biochemical analyses were carried out as part of this study. This investigation was innovative, as no previous studies of this nature had previously been carried out. This study investigated and explored the suggested natural physiological roles of GSTs in black-grass with respect to plant growth and climate

change. The use of GST activity as a marker for predicted herbicide efficacy could lead to the development of a rapid diagnostic kit for herbicide resistance which would be of considerable benefit to farmers when choosing autumn spray programmes.

3. The final stages of this study examined whether resistant and susceptible populations of black-grass differ in respect to growth, GST activity and GSH concentration when subjected to different temperatures, and discusses the implications of the findings for herbicide efficacy and application timing. This study explored in further detail the physiological role of GSTs in black-grass with respect to changing temperature in accordance with those experienced in the present climate and recorded in the previous study. Metabolic plasticity in black-grass has not been widely studied thus this investigation and its findings may contribute further to the novel hypothesis that the natural roles of GSTs in plant defence against stress subsequently render a plant resistance to further stress such as herbicide treatment.

CHAPTER TWO

THE CHARACTERISATION OF THREE BLACK-GRASS POPULATIONS RESISTANT AND SUSCEPTIBLE TO POST-EMERGENT HERBICIDES: A GLASSHOUSE STUDY

2.1. INTRODUCTION

The control and management of black-grass is becoming increasingly complex and challenging, with resistant populations occurring in a number of regions throughout the UK. Before diagnostic techniques for determining resistance can be carried out on uncharacterised black-grass populations, standard resistant and susceptible reference populations must be identified and characterised to be included in every test for comparison as internal standards. Characterisation is necessary in order to appraise the interaction between the weed and herbicides at the whole plant level. This is important as herbicide resistance can be attributed to a number of factors such as biochemical/physiological changes, morphological features or phenological changes (Moss and Rubin, 1993). The knowledge of differences in the resistance status of reference populations is vital when interpreting results for resistance on unknown populations.

The aim of this study was to characterise black-grass resistance by comparing the response of a commercially available susceptible population (Herbiseed) with the 2 current standard susceptible and resistant reference populations (Rothamsted and Peldon) to a range of new and old herbicidal chemistry. This involved 5 commercially available herbicides and 1 experimental herbicide, and characterising resistance by means of studies. The experiments initially examined the morphological response exhibited by each individual population to herbicide treatment under controlled growth conditions. A further aim was to establish whether the Herbiseed population could be used in experiments as a standard susceptible reference population. A "Resistance Rating" was established for each herbicide tested, utilising a new rating system for classifying populations for their degree of resistance to herbicides based on comparisons of herbicide activity with 1 standard reference population devised by Moss *et al.*, (1999).

Resistance was further characterised by means of related biochemical studies of the Herbiseed and Peldon populations through protein content and GST activity determination following herbicide treatment. It is well documented that GSTs play key roles in the detoxification of herbicides (Lamoureux and Rusness, 1989, 1993). This was carried out to provide baseline data from both susceptible and resistant populations, substantiating that herbicide treatment induces GST activity in black-grass and is presented in Appendix 1 for reference.

2.2. MATERIALS AND METHODS

2.2.1. Plant Material.

Three populations of black-grass were used. The susceptible population, Rothamsted, was collected from Broadbalk field at Rothamsted, which has never received herbicide applications. The assistance of Dr S. R. Moss in sampling in the summer of 1997 is acknowledged. This population is currently used as the standard susceptible reference population in the majority of experiments and resistance testing. The second proposed susceptible population, Herbiseed, was purchased from Herbiseed Ltd, Berkshire, UK. The company obtained the original seed stock from a wheat field in Wokingham, Berkshire, UK, which had not been previously treated with herbicides for over 7 generations (Herbiseed Ltd, UK, Personal Communication, 1999). The resistant seed was collected from Hams field at Peldon Hall Farm, Peldon, Essex, UK, again with the assistance of Dr S. R. Moss in 1996. This location is where a high degree of black-grass resistance to CTU was initially detected in the UK in 1984. Since its discovery, this population remains the most resistant to CTU (Moss and Cussans, 1985).

The seeds were sown in a soil-based growing medium (John Innes compost No. 2) with an OM content of 7.25 % mass/mass determined by analysis by ADAS Wolverhampton. The

pots were well watered from below. A high degree of variability was experienced in the establishment of the populations which led to a requirement of extra pots being germinated and plants transplanted in order to provide a uniform population for treatment in each pot. All plants were raised under glasshouse conditions, which allowed some degree of regulation over prevailing environmental conditions. The temperature regulator of the individual glasshouse bay was set to operate a 15°C, 14 h day and 8°C, 10 h night (± 5°C). However, due to increasing temperatures as spring progressed into summer, temperatures regularly reached 25-30°C during the day and dipped to a min of 5°C on cold nights. Supplementary lighting to extend daylight when required early in the experiment was provided by 400 Watt SON/T sodium lamps (Thermoforce, Cumbria, UK) to ensure that photosynthetic photon flux density did not drop below 120μmol m^{-2} sec^{-1}. The maximum flux density of natural daylight was approximately 538 μmol m^{-2} sec^{-1} during the summer months. Plants received a min 14 h photoperiod per day to maximise seed germination and were watered twice on a daily basis via automatic sub-irrigation and light overhead manual watering. Although there were unavoidable environmental fluctuations, both resistant and susceptible plants were grown at the same time so they were exposed to identical environmental growth conditions.

2.2.2. Post-emergent herbicide application.

Six herbicides (Table 2.1) were applied via a custom built Precision Pot Sprayer delivering the equivalent of 200 l ha^{-1} through 2 Lurmark 03 F110 flat fan nozzles (Lurmark, Longstanton, Cambridge, UK) at 7 bar pressure (equivalent to 2-3 bar in the field) in an attempt to mimic field application. The medium quality spray was applied at a height of 45 cm above the pots. The above protocol was in accordance with the guidelines of Moss (1996). Treatments 1-5 were applied at a range of 4 concentrations (0, 0.1, 0.5 and 1.0 Field Rate (FR)) of each individual herbicide for dose-response experiments. Treatment 6 was only applied at FR as, according to Cyanamid (Peter Tayler, Personal Communication,

2000) this was the only rate at which it had been commercially tested. All other remaining plants were treated with tap water as controls. The lowest recommended FR was used as the plants were in optimal growth conditions and therefore optimal herbicide efficacy should have been achieved, as there were no environmental stresses present. In accordance with Moss (1996) no further additives were used.

Table 2.1. Post-emergent Herbicides Used

Trade Name & Formulation	Active Ingredient	Field Rate Dosage kg ai ha^{-1}	Mode of Action
1. Stefes IPU 500 SC	Isoproturon	1.500	Inhibition of photosynthesis at photosystem II
2. Cheetah Super EW	Fenoxaprop-P-ethyl	0.055	Inhibition of acetyl CoA carboxylase ("fop")
3. Topik 240 EC	Clodinafop-propargyl	0.060	Inhibition of acetyl CoA carboxylase ("fop")
4. Checkmate EC	Sethoxydim	0.289	Inhibition of acetyl CoA carboxylase ("dim")
5. Lexus 50 DF WG	Flupyrsulfuron-methyl	0.010	Inhibition of acetolactate synthase
6. AC210 EC	Flufenacet & Pendimethalin	0.024 1.200	Inhibition of cell division & microtubule assembly

To determine the dose-response of black-grass to the herbicides, the 3 populations were sown at 20 seeds in a 5 inch pot. At the 1 leaf stage (GS11), emerging Herbiseed plants were thinned out to leave 5 evenly spaced plants per pot. In the case of Peldon and Rothamsted, where emergence was poor, transplanting surplus plants from other pots of the same population or from reservoir pots was carried out to achieve the desired number of 5 plants per pot. At herbicide application, the plants were at the 2-3 leaf stage (GS12/13) (3 weeks after sowing) and the growth medium was moist, no plants were wilting and there was no surface wetness on the leaves. The pots were placed in a randomised block with 4 replicates including control pots, in the glasshouse. Pots were

spaced in order to prevent leaf-to-leaf transfer of herbicide and were watered regularly from below. Fresh weight of all above ground biomass of every plant from every pot was assessed 2 weeks after herbicide application. The dose-response experiment was carried out twice. The experiment studying the efficacy of AC210 on the 3 populations was carried out separately at a later date due to delays in obtaining the chemical and repairs required on the pot sprayer. This experiment was carried out once only with 8 replicates.

2.2.3. Data Analysis.

Herbicide efficacy was initially expressed as percentage reduction in fresh weight relative to the untreated controls for the same population. Dose-response data was determined from observations of 24 plants per treatment. Dose-response curves and statistical analyses by factorial analysis of variance were calculated and analysed using the statistical package Genstat 5 Release 4.1 (Lawes Agricultural Trust, Rothamsted Experimental Station, UK). In all cases, standard errors were calculated. Examples of original data and worked statistical analyses are presented in Appendix 2.

Using the percentage reduction in fresh weight, a degree of resistance was designated to each population studied with respect to each herbicide applied. This was determined using the new resistance classification system "the "R" system", based on the * rating system, but which only requires the use of a single susceptible standard as proposed by WRAG (Moss *et al.*, 1999), to enable a standardised interpretation of glasshouse screening results. The system has 4 categories: RRR, RR, R? or S (susceptible). The pre-requisite for the susceptible standard is that percentage reduction in foliage fresh weight (control) is greater than 80%. The percentage reduction values between the susceptible standard and zero are then separated into 5 categories. One of the categories of the susceptible range is subdivided into 2 smaller categories at its midpoint – S and 1^{*}. The degree of resistance assigned is determined from the percentage reduction in the foliage fresh weight value for

the susceptible standard, which is obtained for each individual herbicide. This results in the values depicting the categories being different between tests (Moss *et al.*, 1999). An example of this system is presented in Figure 2.1.

For example:

Susceptible standard percentage reduction value = 95%. Each category = 19% (i.e. 95% ÷ 5 = 19%).

Thus <19% = 5*

19% to 38% = 4*

38% to 57% = 3*

57% to 76% = 2*

76% to 85.5% = 1*

85.5% to 95% (and over) is S (susceptible).

SUSCEPTIBLE STANDARD

	S	S	1*	2*	3*	4*	5*
Thus,	95%	86%	76%	57%	38%	19%	0%

i.e. susceptible partially ---------- increasingly resistant ------→ resistant

Testers are then advised to designate samples tested with an R or S rather than a * rating:

5*/4* = RRR Resistance confirmed, highly likely to reduce herbicide performance

3*/2* = RR Resistance confirmed, probably reducing herbicide performance

1* = R? Early indications that resistance may be developing, possibly reducing herbicide performance

S = S Susceptible

Figure 2.1. The "R" system. A new classification system for designating herbicide resistance in grass weed screening assays, taken from Moss *et al.*, (1999).

2.3 RESULTS

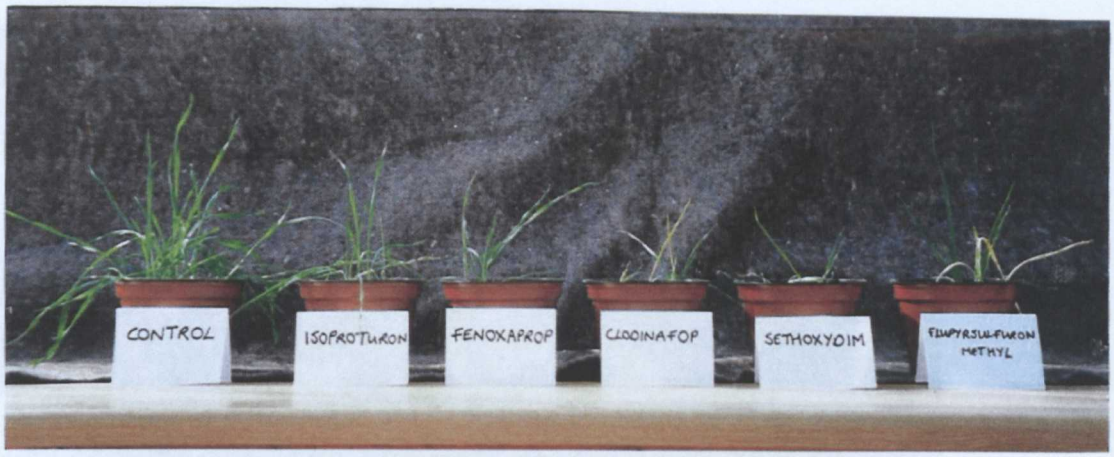
2.3.1. Characterisation of herbicide resistance in black-grass to post-emergent herbicides under glasshouse conditions.

Figures 2.2. and 2.3. illustrate the effect of FR of 5 commercially available and 1 experimental post-emergent herbicide treatments on the growth of 3 differing populations of black-grass under glasshouse conditions. These results are comparable to current findings simulating field conditions (Moss and Clarke, 1995; Willis *et al.*, 1997).

The 2 susceptible populations Herbiseed and Rothamsted exhibited considerable reductions in growth as early as 4 days after treatment (dat). Leaf production and tillering was totally inhibited in most susceptible plants treated with FR and in some cases 0.5 FR. Growth inhibition was the first symptom observed of herbicide treatment. When Peldon plants were treated with the same applications, their growth and development was less affected than that of the 2 susceptible populations, except in the case of treatment with SETH.

Typical symptoms of herbicide action were evident as chlorosis and necrosis from 6 dat in the susceptible populations, as shown in Figure 2.4. However, the effects of IPU and FPM were insufficiently developed in comparison. Chlorosis through loss of photosynthetic pigments spread to affect whole leaves of susceptible plants 10-12 dat with 0.5 and 1.0 FR of all the herbicides. Development of symptoms was initially observed at leaf tips or margins of leaves which were fully expanded at the time of herbicide application. By the time of harvest, the leaves of some susceptible plants had lost all pigmentation and necrosis was evident.

Rothamsted



Scale ◄——► = 10cm

Herbiseed

Scale ◄——► = 10 cm

Peldon

Scale ◄——► = 10cm

Figure 2.2. Effects of post-emergence herbicide treatment on growth of 3 differing populations of black-grass in glasshouse experiments. Photograph taken 14 dat with FR (refer to Table 2.1. for herbicide concentrations).

i) Controls – Peldon, Herbiseed and Rothamsted

Scale ◄——► = 10cm

ii) Treated - Peldon, Herbiseed and Rothamsted

Scale ◄——► = 10cm

Figure 2.3. Effects of AC210 herbicide treatment on growth of 3 differing populations of black-grass in glasshouse experiments. Photograph taken 14 dat with FR (refer to Table 2.1. for herbicide concentrations).

Peldon plants exhibited very few of the above symptoms, as shown in Figure 2.5., except when treated with SETH where herbicide effectiveness was clearly demonstrated by both chlorosis and necrosis. In plants more resistant to herbicide application, the emerging leaf was observed to be green and maturing. In a few cases, particularly those following AC210 applications to Peldon plants, any visible symptoms of chlorosis after application had disappeared by the time of harvest and the plants appeared green and healthy.

Additional effects of herbicide treatment were exhibited through clear structural changes expressed as leaf epinasty and stunting in young, elongating leaves of susceptible plants treated with AC210, as shown in Figure 2.6. These effects were combined in the case of treatment with SETH with visible anthocyanin accumulation (reddening of stems) in all 3 populations as demonstrated in Figure 2.7.

i) Rothamsted

Scale ◄—► = 10cm

ii) Herbiseed

Scale ◄—► = 10cm

Figure 2.4. Effects of SETH (i - Rothamsted) and CP (ii – Herbiseed) on growth of black-grass in glasshouse experiments. Photograph taken 14 dat with rates shown as percentage of FR (0.2895 kg ai ha^{-1} and 0.06 kg ai ha^{-1}, respectively).

Scale ◄——► = 10 cm

Figure 2.5. Effects of FE on growth of the black-grass population Peldon in glasshouse experiments. Photograph taken 14 dat with rates shown as percentage of FR (0.055 kg ai ha^{-1}). No visible symptoms of herbicide treatment were evident.

1 2 3 4 5 6

Scale ◄——► = 10cm

Figure 2.6. Effects of AC210 on growth of black-grass in glasshouse experiments. Photograph taken 14 dat with FR (1.224 kg ai ha^{-1}). Plants are exhibited from the left:

1. Peldon control; 2. treated
3. Herbiseed control; 4. treated
5. Rothamsted control; 6. treated

Note characteristic symptoms of plant stunting, leaf epinasty and developing chlorosis.

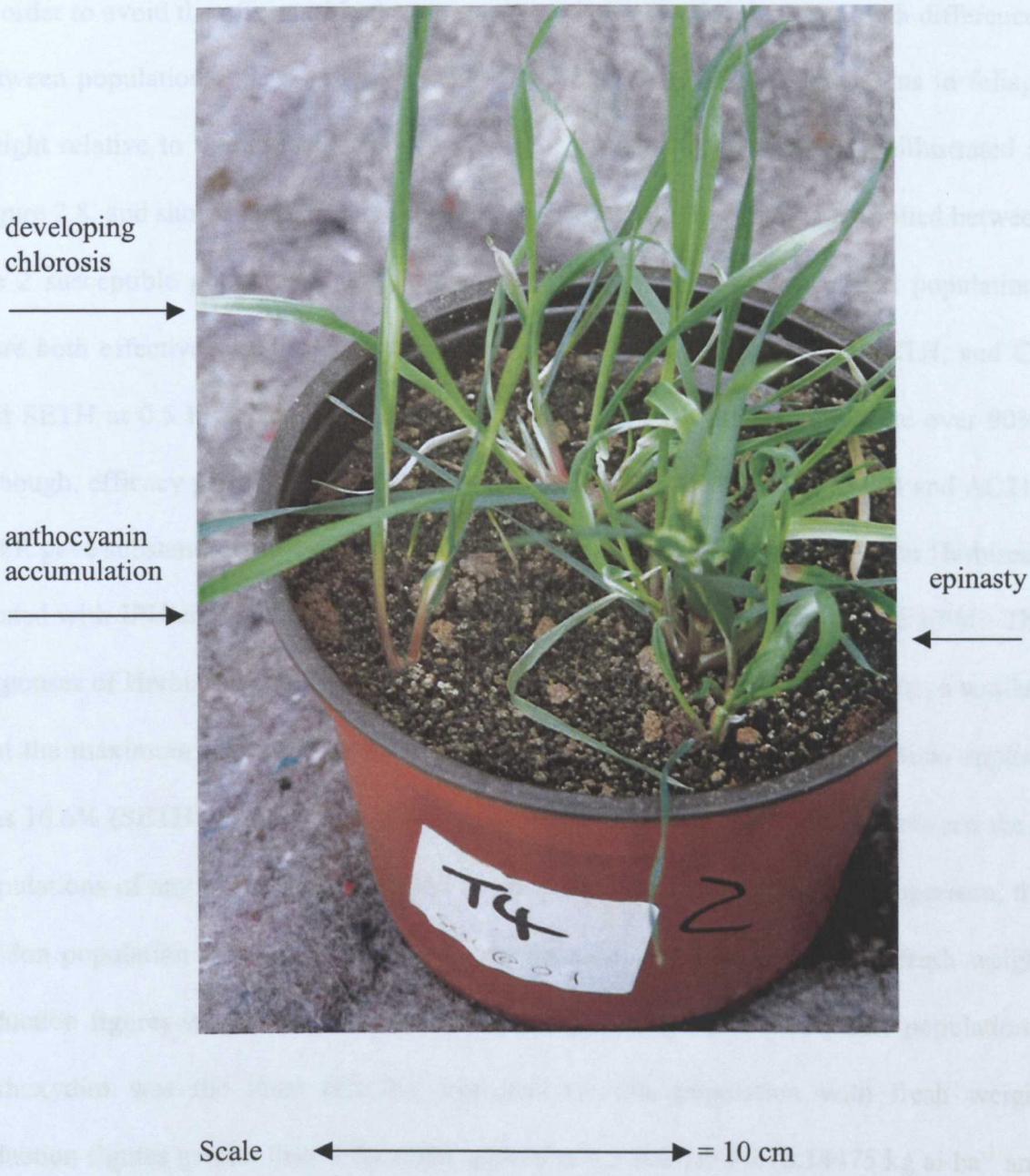


Figure 2.7. Effects of SETH on growth of the black-grass population Herbiseed in glasshouse experiments. Photograph taken 14 dat with 0.1 FR (0.02895 kg ai ha^{-1}). Note typical symptoms of leaf epinasty, anthocyanin accumulation and developing chlorosis.

2.3.2. The effect of increasing doses of herbicide treatment on three differing populations of black-grass.

In order to avoid the effects of herbicide treatment being confused with growth differences between populations, the data was initially converted to percentage reductions in foliage weight relative to the untreated for the same population. These results are illustrated in Figure 2.8. and show marked differences in susceptibility to the herbicides applied between the 2 susceptible populations and Peldon. The Herbiseed and Rothamsted populations were both effectively controlled by the recommended FR of FE, CP and SETH; and CP and SETH at 0.5 FR. The percentage reduction figures in fresh weight were over 90%, although, efficacy predictably tailed off at lower rates. In contrast, IPU, FPM and AC210 at FR gave substantially less control with minimum reduction figures of 52% for Herbiseed treated with IPU and maximum reduction of 72% for Rothamsted treated with FPM. The responses of Herbiseed and Rothamsted in terms of fresh weight reduction were so similar, that the maximum difference between the 2 populations at any rate of chemical applied was 16.6% (SETH at 0.1 FR – 0.02895 kg ai ha^{-1}). The largest difference between the 2 populations of any chemical applied at FR was only 6.29% (AC210). In comparison, the Peldon population responded to herbicide application, but with percentage fresh weight reduction figures substantially less than the other 2, clearly more susceptible populations. Sethoxydim was the most effective chemical on this population with fresh weight reduction figures greater than 93% when applied at 0.5 and 1.0 FR (0.14475 kg ai ha^{-1} and 0.2895 kg ai ha^{-1}, respectively). Efficacy of the other herbicides at FR did not attain a level of fresh weight reduction above 45% with minimum control of 2.5% for IPU at 1.5 kg ai ha^{-1}.

Analysis of variance indicated a highly significant difference between treatments,

populations and rates (P = <0.001) bar 2 exceptions. These being (i) there was no difference between the response of the populations (P = >0.05) treated with SETH and (ii) there was no difference between the rates applied to the different populations of FPM (P = >0.05). Across the whole trial, there were highly significant interactions (P = <0.001) between treatments and populations, in that there was a difference between the effects of each treatment on each population. A coefficient of variation (CV) of 18.1% was recorded which is acceptable for an experiment of this nature.

Figure 2.9. presents dose-response curves obtained from applying herbicides at increasing dose rates to 3 populations of black-grass. Factorial dose-response analysis of variance on the individual dose responses to each herbicide indicated highly significant differences between the yield responses of the 3 populations (P = <0.001), except for SETH where they were similar (Figure 2.9). There were also highly significant differences between the rates of each herbicide applied (P = <0.001) with the exception of FPM, where there was no distinguishable difference shown between applying increasing dose rates to the 3 populations. All 3 populations exhibited highly significant linear relationships with dose rate of herbicide (P = <0.001) i.e. as dose increased, fresh weight (g) decreased except for treatment with FPM where the relationship was much less pronounced and insignificant (P>0.05). Further analysis indicated significant interactions (P= <0.022) between population and rate for IPU, FE and CP i.e. the effect of increasing dose was different on each population. This was not relevant however for SETH and FPM as demonstrated in Figure 2.9. the populations responded very similarly to each dose.

Figure 2.9. provides further confirmation that the Herbiseed and Rothamsted populations responded very similarly to the herbicides. The Peldon population exhibited less pronounced linear relationships with dose to every herbicide except SETH where it was

very similar to both Rothamsted and Herbiseed. Factorial dose-response analysis of variance was carried out to clarify the correlation between the responses of Herbiseed and Rothamsted. These analyses also suggested that the difference between the populations and their interactions with dose rate shown earlier was due to Peldon being more resistant to the chemicals applied.

Statistical analysis revealed significant differences ($P = <0.05$) when comparing the yield responses of Herbiseed and Rothamsted, except for CP. There were also highly significant differences between the rates ($P = <0.001$) applied to the 2 populations except as before for FPM. Both populations exhibited highly significant linear relationships to dose ($P = <0.001$) bar FPM. The most important factor of these analyses was that they indicated that there was no significant interaction between population and rate, therefore indicating that there was a correlation between the 2 populations and the way that they responded to each treatment and dose rate applied. These results confirmed the Peldon population as much more resistant than the other 2 to the various MOA studied particularly by demonstrating such small variations in fresh weight response between differing doses of most of the herbicides applied.

In summary, as dose rate increased, fresh weight (g) and percentage fresh weight reduction decreased and therefore herbicide efficacy was observed to decrease. These results were all comparable to those of Moss and Clarke (1995).

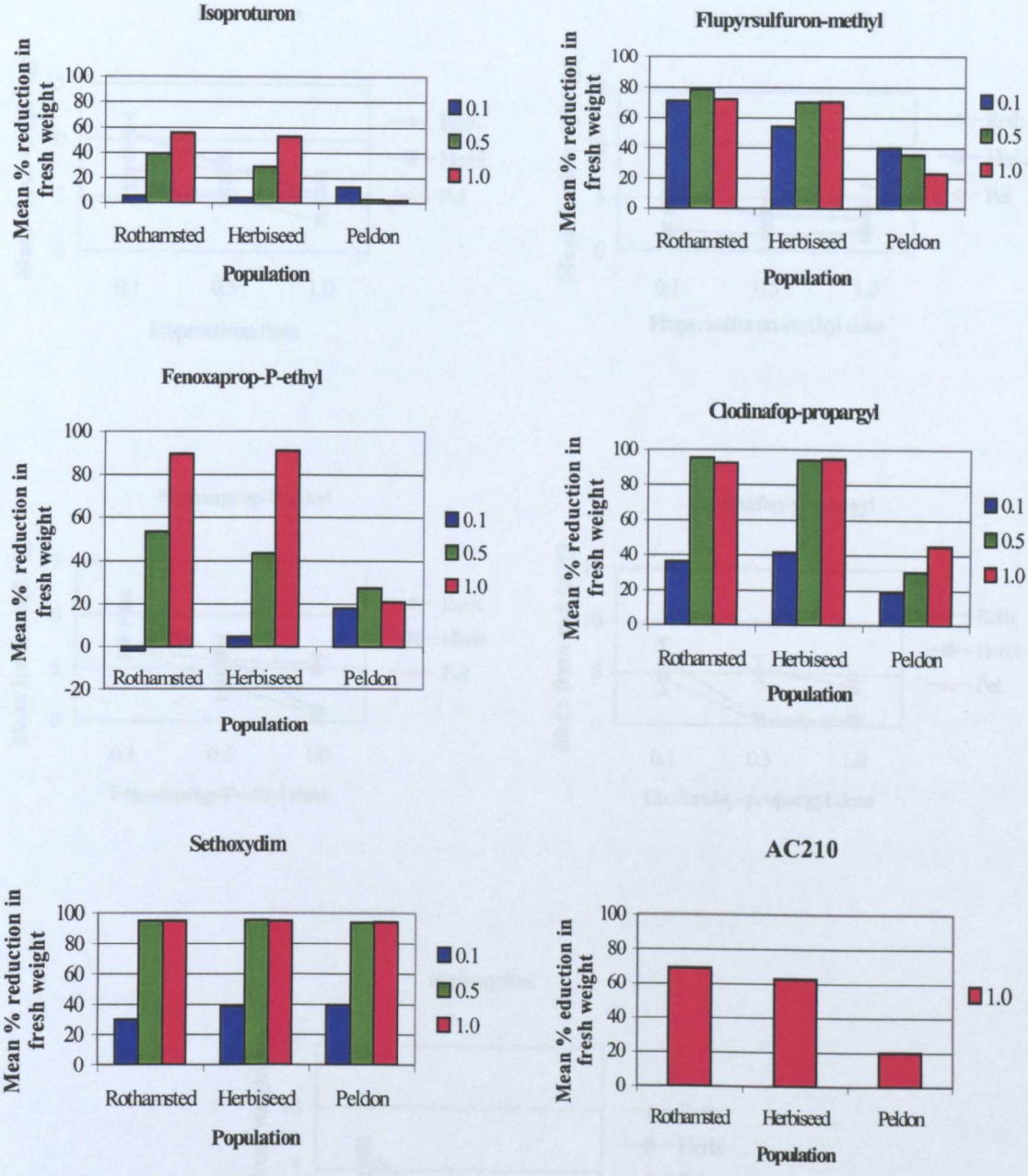


Figure 2.8. Efficacy of post-emergent herbicide treatment on fresh weight of 3 differing populations of black-grass 14 dat in glasshouse experiments. Reductions in fresh weight are given as a percentage of the untreated means. Herbicide concentrations are shown as percentage of FR as shown in Table 2.1. The graph illustrates combined data from 3 experiments, where each value is a mean of 8 pots, each containing 5 plants i.e. n = 40 plants. **Key to abbreviations:** *Herbicide doses*: 0.1, 0.5 and 1.0 FR of the individual herbicides.

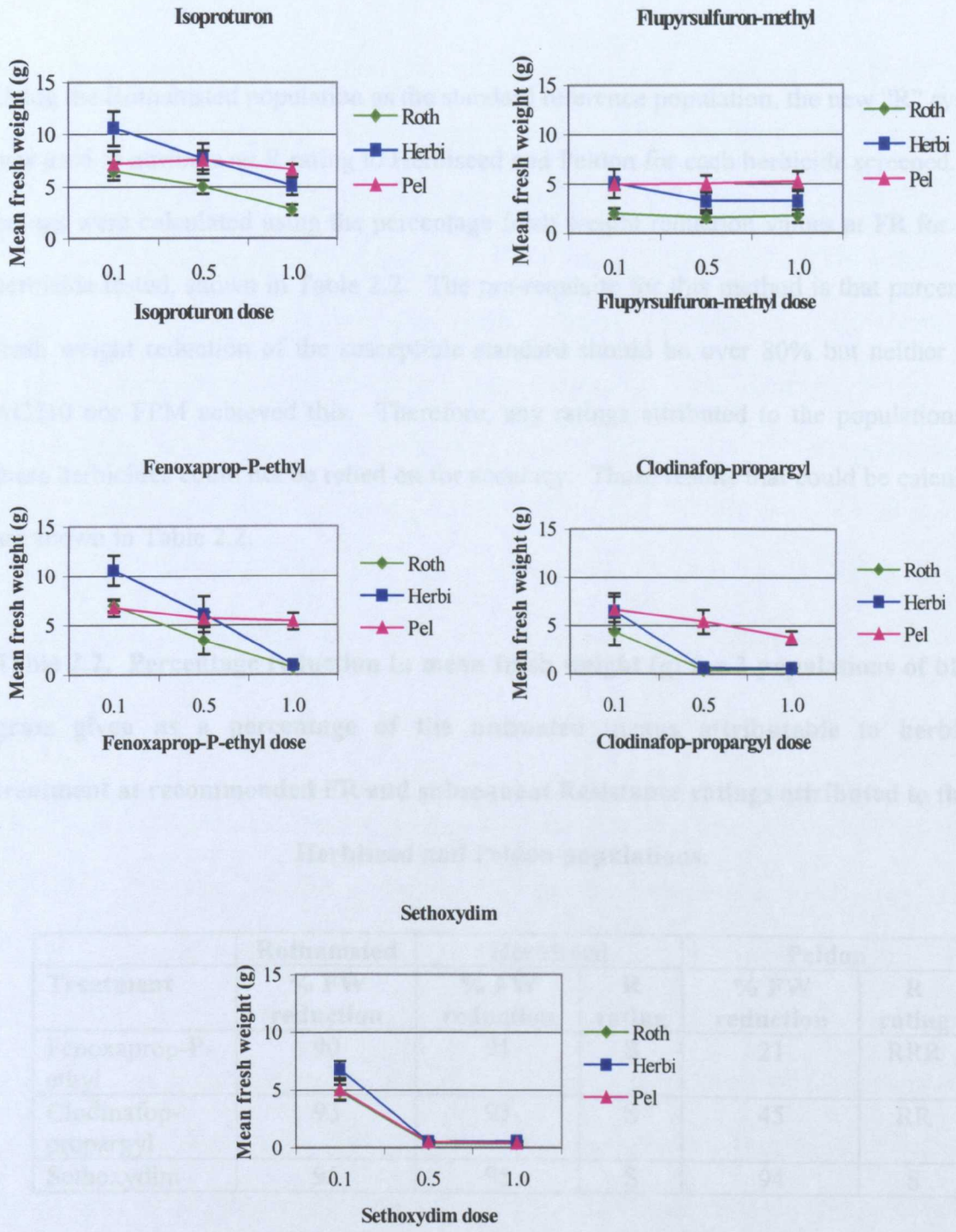


Figure 2.9. Effects of increasing post-emergent herbicide dose on the fresh weight of 3 differing populations of black-grass 14 dat in glasshouse experiments. Herbicide concentrations are shown as multiples of FR as shown in Table 2.1. The graph illustrates combined data from 2 experiments, where each value is a mean of 8 pots, each containing 5 plants, i.e. n = 40 plants. **Key to abbreviations**: ***Herbicide doses*****:** 0.1, 0.5 and 1.0 FR. ***Populations*****:** Roth – Rothamsted, Herbi – Herbiseed and Pel – Peldon.

2.3.3. Designation of "The "R" System" to resistant and susceptible black-grass.

Using the Rothamsted population as the standard reference population, the new "R" system was used to attribute an R rating to Herbiseed and Peldon for each herbicide screened. The ratings were calculated using the percentage fresh weight reduction values at FR for each herbicide tested, shown in Table 2.2. The pre-requisite for this method is that percentage fresh weight reduction of the susceptible standard should be over 80% but neither IPU, AC210 nor FPM achieved this. Therefore, any ratings attributed to the populations for these herbicides could not be relied on for accuracy. Those results that could be calculated are shown in Table 2.2.

Table 2.2. Percentage reduction in mean fresh weight (g) for 3 populations of black-grass given as a percentage of the untreated means attributable to herbicide treatment at recommended FR and subsequent Resistance ratings attributed to the Herbiseed and Peldon populations.

	Rothamsted	Herbiseed		Peldon	
Treatment	**% FW reduction**	**% FW reduction**	**R rating**	**% FW reduction**	**R rating**
Fenoxaprop-P-ethyl	90	91	S	21	RRR
Clodinafop-propargyl	93	95	S	45	RR
Sethoxydim	95	95	S	94	S

Key: FW – fresh weight

2.4. DISCUSSION

The principal aim of these experiments was to characterise resistance in 3 black-grass populations. The glasshouse dose-response experiments confirmed that both the Rothamsted and Herbiseed populations were equally susceptible to FE, CP and SETH at both 0.5 and 1.0 FR, as shown in Figure 2.9. Their susceptibility to IPU, FPM and AC210 was much less pronounced, but they shared similar responses to these chemicals. However, it cannot be speculated that these populations are identical, as they are genetically different. These results agree with those of Cocker *et al.*, (1999) and Moss and Clarke (1995). However, the results for IPU, FPM and AC210 were not as clear in comparison to those of (Moss and Clarke, 1992; Teaney *et al.*, 1995) on susceptible populations. This may indicate a difference in experimental methodology or herbicide application.

The Peldon population exhibited differing resistance to each chemical with the exception of SETH. The results shown in Figures 2.8. and 2.9. demonstrate clearly that cross-resistance was exhibited with obvious lack of control by IPU and moderate control by FE and CP. This study confirms that while resistance does not cause complete inactivity of herbicides, substantial reduction in activity can occur, in agreement with Clarke and Moss (1991) and Moss and Clarke (1995). However, the scale of the reductions in herbicide performance varies substantially between herbicides for reasons that are at present poorly understood. These results confirm the observations of Moss and Clarke (1995) that there is no consistent relationship between the degree of resistance to FE and IPU in the Peldon population.

There was no evidence of differential efficacy between all 3 populations when treated with SETH at 0.5 and 1.0 FR as exhibited in Figure 2.9., hence a susceptible resistance rating

being attributed to them. This is consistent with unpublished glasshouse studies carried out by Clarke and Moss (1991). However, it was also clear that small contributions from several factors might combine to result in poor herbicide performance.

The second stage of this study assessed the effect of herbicide treatment on the Herbiseed and Peldon populations. Where possible, resistance ratings were attributed to Herbiseed and Peldon with respect to the individual herbicides applied. These were useful and novel observations for both populations as this new classification has only just been adopted. These results and R ratings clearly confirm the observations of Moss and Clarke (1995) that relating resistance to chemical class is misleading. There is a need to consider herbicides individually, as resistance may occur to some, but not necessarily all herbicides within a class, as illustrated for FE, CP and SETH in Figures 2.8. and 2.9 and Table 2.2. No resistance ratings were attributed to Herbiseed and Peldon for IPU, FPM and AC210 due to the percentage reduction in fresh weight of the Rothamsted population not being high enough to calculate them. The reductions in herbicide efficacy that led to this could be due to various reasons such as glasshouse conditions and herbicide application. The new resistance rating system utilised in this study carries a degree of risk as the higher the degree of resistance, the greater risk of herbicide failure. This was the additional reason why no ratings were attributed for the 3 chemicals to either population.

The response of plants varies with growth parameters and experimental procedures. However, if resistant and susceptible populations are grown alongside each other simultaneously as in this study, variation should be minimised. Variations in fresh weight values were observed, which may be due to the experiments being carried out from early spring through to mid-summer. The night temperature in the initial experiment regularly dropped to the min bay setting of 5°C that may go some way to explaining the variation experienced with germination and subsequent vigour of the populations. Daytime

temperatures were observed to reach a maximum between 30-32°C. Germination pre-requisites for black-grass requires a wide temperature range between 10-25°C with an optimum of 15°C. Temperatures below 3°C and above 30°C are known to disrupt germination (Froud-Williams, 1985). This may explain the poor germination exhibited by the Rothamsted and Peldon populations. Seed vigour must also be taken into account as the Rothamsted and Peldon seed stocks were older than that of Herbiseed.

In contrast to Moss and Clarke (1992) and Teaney *et al.*, (1995), a different growing medium was used here and all plants were harvested 14 dat for uniformity. It is well documented that substituted urea herbicides take longer to work, as they are residual acting as opposed to contact. Foliage herbicides are absorbed over a period ranging from minutes to days, but soil applied herbicides such as IPU can take considerably longer. Consequently, considerable time may be taken for the lethal herbicide concentration to accumulate, especially in parts of the plant far from the site of herbicide application (Devine, Duke and Fedtke, 1993). Moss (1996) suggests that plant harvest in glasshouse experiments should be when herbicidal effects are obvious – usually 3-4 weeks. In this study, there were clear herbicidal effects 2 wks after treatment. This was probably due to high temperatures increasing translocation of the herbicides throughout the plants and increased herbicide metabolism. Therefore, they were harvested at this time. However, it may be that leaving the plants longer may have allowed IPU, FPM and AC210 to have had a greater effect on the susceptible populations, whereas on the other hand a longer period may have resulted in regrowth. It is well documented that applications of FPM and AC210 lead to growth of weeds being inhibited in hours. However, visible symptoms in some weed species may take between 4 wks and 3 mths to appear (Dupont, 1998; Peter Tayler, Cyanamid, Personal Communication, 2000). In addition, the ideal OM content of the growing medium should be within a range of 3-10%, ideally about 5% (Moss, 1996). Analysis of the growing medium used in this study indicated an OM content of 7.25%

mass/mass. It is well documented that IPU efficacy drops in soils of OM content over 10% (Blair, 1985). This was a relatively high result, which may have contributed to its reduced efficacy in this study. It must also be considered that IPU was applied at the newly revised recommended rate of 1.5 kg ai ha^{-1} and compared to the results of Moss and Clarke (1995), it was not as effective as the full dose rate of 2.5 kg ai ha^{-1}. This suggests that IPU applied at 1.5 kg ai ha^{-1} should not used as a stand-alone product but be used as part of a sequence or in mixture.

The competitive ability of weeds and efficacy of resistance mechanisms ensure that resistant populations survive in the field. Devine *et al.*, (1993) suggest that a degree of resistance will be selected for if there are any small inherited changes in plant morphology. Sharples *et al.*, (1997) indicate that although there are marked differences between black-grass populations in terms of resistance, these are not associated with differences in biological fitness as resistant biotypes have similar patterns of growth to susceptible biotypes. Visual comparisons between resistant and susceptible populations in this study indicated no differences in relative growth rate or plant fitness. Differences were evident in terms of fresh weight response to the herbicides applied as can be seen in Figure 2.8. These observations confirm those of Kemp *et al.*, (1990), that there is no difference in growth between resistant and susceptible plants indicating that resistance mechanisms reside at the biochemical level. Characterisation of resistance in susceptible populations is currently not a widely explored area, yet is critical when interpreting small differences in responses between populations at both whole plant and biochemical levels. As indicated by Cocker *et al.*, (1999), small insignificant differences may become larger and of more practical significance with continued selection processes.

Herbicide efficacy is reliant on the amount of active ingredient reaching the target site within the plant rather than the amount actually applied (Blair, 1978). This is regulated by

a series of complex interactions between the herbicide applied, target weed species, the soil and prevailing environmental conditions. The herbicide requires an actively growing plant and the environmental factors of temperature, frost, relative humidity, soil type, OM and rainfall in the field will exert influences on plant growth and herbicide toxicity as illustrated by this study. Dry or cold conditions are well known to reduce or slow down the efficacy of many chemicals, as temperature is known to influence metabolic processes within the plant and affect rates of herbicide metabolism as demonstrated by these results.

Plants respond differently to herbicide treatment, for example, PSII inhibitors cause desiccation effects, which are not induced by herbicides from different classes. This can render results interpretation slightly hazardous. The efficacy of the different herbicides applied during this study varied mainly due to variations in glasshouse conditions. However, high temperatures did not significantly increase the efficacy of IPU as suggested in Chapter 1. Treatment by IPU initially results in rapid cessation of plant growth followed by developing chlorosis and subsequently necrosis as observed in some plants of the susceptible populations as can be seen in Figure 2.2. Plant injury observed in plants treated with FE, CP and SETH was similar as illustrated in Figures 2.2., 2.4. and 2.7., which is of no surprise as they share the same TS, but CHDs tend to have slower rates of penetration into the leaves (Cobb, 1992).

In this study there was evidence of SETH displaying anti-auxin activity in young, rapidly elongating tissues resulting in significant stunting of plants compared to the controls as illustrated in Figure 2.7. As a foliar acting herbicide it initially comes into contact with the cell membrane before intracellular metabolism and translocation to the grass meristem. This provides the opportunity for this chemical to exhibit *in vivo* anti-auxin activity in addition to inhibition of ACCase, inducing a plant response similar to that induced by auxin-type herbicides. This was in the form of major morphological growth changes

visible a week after application expressed as the characteristic symptoms of leaf epinasty as demonstrated in Figure 2.7. This is in response to ethylene evolution and abnormal apical growth whereby plant tissue responds to an external stimulus leading to increased growth on one side of any organ causing bending in young tissues (Cobb, 1992).

Efficacy of FPM is a result of ALS inhibition coupled with a varying ability to metabolise the chemical. Hence there is a correlation between the rate at which a plant metabolises it and its tolerance or susceptibility. This was demonstrated by susceptible black-grass metabolising FPM more slowly (half-life of 20 h) than more tolerant wheat and *Avena fatua* species (half life ≤ 2 hours) (Koeppe *et al.*, 1998). Symptoms of treatment are very similar to other ALS inhibitors in that initially there is rapid cessation of growth in susceptible species. Anthocyanin accumulation is also an associated symptom although this was not observed in this study (Cobb, 1992). The time taken for symptoms of herbicide treatment to appear and death to occur may vary between species, which was a factor in this study.

AC210 is a mixture of old and new chemistry in the form of pendimethalin and flufenacet. Pendimethalin is a residual, selective herbicide, which both inhibits microtubule formation and causes microfibril disorientation. As a chemical, it is strongly absorbed to soil, therefore high OM content is important (Cyanamid, 1997) which was a factor in this study. Little information is currently available about flufenacet except that it is similar to pendimethalin in its MOA of inhibiting both cell growth and division. As a formulation, AC210 is documented to be extremely slow acting with no effects visible for up to 3 months after which control of susceptible populations is good. This partly explains the lack of efficacy shown by this chemical in this study. In addition, herbicidal efficacy of this compound is maximised against very young grass weeds at the 1-2 leaf stage (Peter Tayler, Cyanamid, Personal Communication, 2000). This study involved herbicide

application at the 2-3 leaf stage, indicating that efficacy of this compound was therefore compromised. The incorporation of pendimethalin into this formulation meant that the Peldon population due to enhanced metabolism exhibited partial resistance to AC210.

This investigation presents novel observations, however, further study is required to clarify these. No reference TS or multiple resistant populations e.g. Oxford and Lincs E1, were included in the study due to space and time limitations. Therefore, any resistance traits towards TS herbicides were not accounted for in the Herbiseed population. It is postulated that from these results, no resistance traits would be found if tested. To confirm this further study incorporating the use of other populations is required. It is also suggested that the experiments be repeated with IPU, FPM and AC210 allowing more time for the herbicide in question to work. This in turn would produce improved efficacy results and thus resistance ratings could be attributed for the populations to these chemicals.

2.5. CONCLUSION

Herbicide resistance was characterised in 3 black-grass populations in a series of glasshouse experiments. The Herbiseed population may be used as a standard susceptible reference population in conjunction with the Rothamsted population, when testing unknown populations against FE, CP and SETH. Novel resistance ratings have been applied to this population for future reference.

CHAPTER THREE

A DEVELOPMENTAL STUDY OF GLUTATHIONE *S*-TRANSFERASE ACTIVITY IN BLACK-GRASS IN THE FIELD

3.1. INTRODUCTION

Herbicide resistance in black-grass is an ongoing problem. There is clear evidence to suggest that more than one resistance mechanism exists in black-grass, with GSTs implicated as partly responsible for resistance based on enhanced metabolism to certain herbicides (Cummins *et al.*, 1997a; Hall, Moss and Powles, 1997; Reade *et al.*, 1997). There is widespread acknowledgement that GSTs metabolise herbicides and many studies have been carried out investigating the activity of this detoxification enzyme with respect to herbicides and other exogenous stressing agents (Marrs, 1996). Sharples, Hull and Cobb, (1995) and Reade *et al.*, (1997) have indicated that the resistant black-grass population Peldon contains approximately double the GST activity of susceptible populations and have suggested that there is a correlation between GST activity and herbicide resistance.

This investigation has examined herbicide resistance in 5 UK black-grass populations in the field with respect to GST activity. The aim was to investigate the relationship between GST activity and black-grass growth and development with the overall aim of testing GST activity as a marker for predicting herbicide efficacy. Commercial cereal field trial observations of resistant black-grass populations indicate that there is a decrease in herbicide efficacy from winter to spring, shown by studying the effect of application timings of fenoxaprop-P-ethyl (Mills and Ryan, 1995; Ryan and Mills, 1997). Therefore, it is important to establish at what growth stage herbicide efficacy is likely to decline. Such an assessment of GST activity might form the basis of a simple test for herbicide resistance and, in addition could potentially be useful as a marker for identifying spray windows for maximum herbicide efficacy in the control of susceptible and resistant black-grass populations.

Untreated black-grass populations in ongoing long-term field trials at sites with confirmed resistant populations have been studied. A sampling strategy was developed to establish GST activity in untreated plants over the winter to spring period for 2 consecutive years testing the null hypothesis that there was no relationship between GST activity and black-grass leaf and plant maturity.

3.2. MATERIALS AND METHODS

3.2.1 Trial Sites.

In an attempt to identify potential herbicide-based resistance management strategies through characterisation studies of herbicide resistance development in black-grass plants in the field, ongoing long-term field studies have been conducted by Syngenta Crop Protection UK Ltd, to which access was given. Black-grass plants were sampled from 5 sites situated in the East of England, in southern and northern Northamptonshire and on the North Northamptonshire/Cambridgeshire border. The details of each site are presented in Table 3.1. No standard susceptible site was included for comparison as it would be impossible to identify a completely susceptible field site, as there would always be a mix of resistant and susceptible individuals present.

3.2.2. Plant Material: A Sampling Strategy.

Five differing black-grass populations were sampled at different growth stages during the winter to spring period of 2 consecutive years. A sampling strategy was developed to establish GST activity in untreated black-grass plants from the trials providing data for both individual leaves and whole plants.

1998/1999.

Whole black-grass plants (n = 5) were randomly sampled from untreated plots of 2 cereal field-trial sites, situated in North Northamptonshire (Site 1) and on the North Northamptonshire/ Cambridgeshire border (Site 2).

1999/2000.

Whole black-grass plants (n = 10) were randomly sampled from untreated plots of 3 cereal field-trial sites, situated in North Northamptonshire (Site 3 which was in a different part of the same field as Site 1), North Northamptonshire/Cambridgeshire border (Site 4) and South Northamptonshire (Site 5). The sample size during this year of study was enlarged to 10 to increase validity.

All the sites sampled had a clay-loam based soil and were routinely ploughed or minimally cultivated for winter cereal cropping. Documentation and information with respect to each sampling was recorded and kept on a sheet similar to that shown in Figure 3.1. During the second year of study, population counts were also conducted.

Whole plants at different growth stages were hand harvested by passing up and down the length and approximately through the centre of the trial plots. A 50 x 50 cm^2 quadrat was randomly thrown to the left of the body and 1 plant was sampled from the bottom left hand corner of the quadrat each time it was thrown. Whole plants, including roots with some surrounding soil to maintain plant structure, were harvested *in situ* using a trowel and individually bagged. Plants were transported back to the laboratory unfrozen (to prevent individual leaves attaching to each other), but kept cool on top of dry ice to prevent wilting. On the same day plants were washed to remove surrounding soil, growth stage recorded and all tillers were subsequently discarded. Each fully expanded leaf was excised from the main shoot using a scalpel and individual leaf fresh weights recorded.

As plants matured, older leaves were observed to be senescing. Using a magnifying glass, the plant leaf base was identified and senesced leaves were regarded as the first fully expanded and hence the oldest leaf on the plant, from which other leaves were sequentially identified. Foliage was frozen in liquid nitrogen and stored at -80°C until required for

protein and enzyme analysis. This sampling strategy was carried out at Sites 1 and 2 four times between February and May 1999, 5 times at Sites 3 and 4 between December 1999 and May 2000 and 10 times at Sites 5 between October 1999 and May 2000. Differences in starting dates for sampling were due to differences in black-grass germination and emergence between sites. The growth stages (Stauss, 1994) identified during this study were:

GS11 - 13 between 1 and 3 true leaves

GS22 – 29 between 2 and 9 tillers

GS30 beginning of stem elongation

GS31 first node at least 1cm above tillering node

GS45/6 late boot stage: flag leaf sheath swollen

GS48 flag leaf sheath opening

GS57 70% inflorescence emerged.

SAMPLING INFORMATION

Experiment No ----------

Trial Location ----------

Date ----------

Type of Sample ----------

Sample No	Treatment/Plot	Growth Stage	Pop^n Counts	Notes

Figure 3.1. Documentation sheet on to which all sampling information was recorded for each sampling date.

Table 3.1. Site details of the five cereal field trial sites sampled during the two year study. (Information provided courtesy of Syngenta Crop Protection UK Ltd). N/A = information not available.

	YEAR	Sites 1 and 3	Site 2	Site 4	Site 5
Trial Location		North Northamptonshire	North Northamptonshire /Cambridgeshire border	North Northamptonshire /Cambridgeshire	South Northamptonshire
Nearest Meteorological Station		Corby, Northamptonshire	Peterborough, Cambridgeshire	Peterborough, Cambridgeshire	Banbury, Oxfordshire
Soil Type		Clay	Clay	Clay	Clay
Crop	1	Winter Wheat	Winter Barley	Winter Barley	Winter Wheat
	2	Winter Wheat	N/A	Winter Wheat	Winter Wheat
Variety	1	Consort	Halcyon	Regina	Madrigal
	2	Consort	N/A	Equinox	Claire
Soil Preparation Method	1	Ploughed	Minimal Cultivation	Minimal Cultivation	Plough
	2	Ploughed	N/A	Plough	Minimal cultivation
Planting Method	1	Drill	Drill	Drill	Drill
	2	Drill	Drill	Drill	Drill
Drill Date	1	N/A	25/10/1998	19/10/98	20/09/98
	2	03/10/99	N/A	26/10/99	19/09/99
Resistance Information		Metabolic resistance to chlorotoluron and fenoxaprop-P-ethyl. Indications of low level target site resistance	Metabolic resistance suspected.	Metabolic resistance suspected.	High levels of metabolic and target site resistance.
Further Information		Continuous wheat rotation for a number of years and is ploughed annually.	Wheat, Wheat, Barley rotation.	Wheat, Wheat, Barley rotation. In previous years has been minimally cultivated with trash left on the soil surface.	Wheat, Wheat, Rape rotation. Previously ploughed annually. Minimal cultivation has resulted in large black-grass populations.

3.2.3. Effect of crop and weed competition on untreated black-grass populations at four differing sites.

Visual observations assessing crop and weed competition and population variation over the winter to spring period were carried out. Ten population counts were carried out per plot by the random dropping of a 50 x 50cm^2 quadrat. These figures were adjusted to give a mean population count of black-grass plants m^{-2}.

3.2.4. Determination of protein content in untreated black-grass leaves sampled from 5 differing cereal field-trial sites over 2 consecutive years.

3.2.4.1. Plant Material. The tissue harvested and separated into individual leaves was subsequently used for protein determination.

3.2.4.2. Preparation of cell-free extracts. Cell-free extracts were prepared using the method of Reade and Cobb (1999). All chemicals were obtained from Sigma (Sigma-Aldrich Company Ltd, Dorset, England) unless otherwise stated. Approximately 0.5g sub-sample accurately weighed of frozen plant tissue of each plant harvested was ground to a fine powder in liquid nitrogen in a pestle and mortar. In many cases individual leaves excised from the main shoot of harvested plants weighed considerably less than 0.5g. Therefore, the fresh weight subsample, which was used in the extraction process, was the whole excised leaf, which had been accurately weighed before analysis began. The powder was thawed in a centrifuge tube containing 5ml of chilled extraction medium (100mM potassium phosphate buffer pH 7.0 containing 5mM diethylenetriaminepentaacetic acid (DTPA) and 10mM sodium ascorbate). Polyvinylpolypyrrolidone (PVPP) at 40g litre^{-1} was added prior to the extract being added, to inhibit polyphenoloxidase activity. The contents of the tube were then homogenised for 20 sec using a Silverson SL2 laboratory homogeniser (Silverson Machines, Chesham,

Buckinghamshire, UK) at full speed. The resulting homogenate was centrifuged (Beckman Avanti 30) at 15000g for 15 min at 4°C and the supernatant desalted on a Sephadex G-25 PD-10 column (Amersham Pharmacia Biotech Ltd, Hertfordshire, UK) by loading 2.5ml onto the column after equilibration with 25ml 0.1M potassium phosphate buffer (pH 7.0) containing 0.25mM DTPA. This was eluted with 3.5ml of equilibrium buffer and the sample collected was the cell-free extract used in all further analyses.

3.2.4.3. Protein determination. The protein content of each extract was determined using a modification of the Bradford (1976) dye-binding assay. Protein reagent stock solution was prepared by dissolving 0.2g of Coomassie Brilliant Blue G-250 (Sigma) in 50ml of 95% (v/v) aqueous ethanol. To this, 100ml 85% (w/v) aqueous phosphoric acid was added creating an exothermic reaction.

The reagent working solution was prepared by taking 15ml of previously prepared stock solution and to this adding 1ml of 0.3% (w/v) sodium dodecyl sulphate (SDS) and distilled water to produce a final solution volume of 100ml. A standard curve was determined by using bovine serum albumin fraction V (Sigma) (BSA) at a range of concentrations (0-20 $\mu g\ ml^{-1}$). Differing concentrations of BSA were increased to a final volume of 200µl through the addition of distilled water. To this, 1ml of reagent was added, vortexed and incubated at room temperature for 3 min, after which the absorbance at 595nm was measured. This was repeated 3 times for each BSA concentration and a linear standard curve obtained. The assay was carried out on each cell-free extract using up to 200 µl of extract, making the final assay volume up to 200 µl with distilled water where required, to which 1ml of reagent was added. The cell-free extracts were diluted where necessary so that all absorbance readings fell within the standard curve.

3.2.5. Determination of glutathione *S*-transferase activity in 5 differing black-grass populations in the field and the relationship between GST activity and black-grass growth and development.

The desalted leaf extracts were prepared for GST assays using the artificial substrate CDNB, following the method of Reade and Cobb (1999). The final assay volume of 1ml was made up of 750µl 100mM potassium phosphate buffer pH 6.5 containing 0.2mM DTPA, 100µl 1mM GSH, 100µl 1mM CDNB to which 50µl black-grass crude enzyme extract was added. Conjugate formation was monitored as the increase in absorption at 340nm measured spectrophotometrically at 20°C and charted on a recorder with paper speed set at 20 mm min^{-1}. Non-enzymatic conjugate formation was determined by replacing the plant extract with an equal volume of assay buffer. To improve this method the assay was adapted by Dr J. P. H. Reade to a Benchmark Microplate reader (Benchmark Microplate Manager, Version 5.0.1 Build 52, 1999, Bio Rad Life Science Group, California) to increase throughput of samples. Due to an inability to obtain an accurate pathlength in the microplate reader, microplate data was quantified using a known concentration of CDNB/GSH conjugate. The final assay volume was scaled down from 1ml to 200µl, made up of the same components as before with variations in buffer volume depending on the volume (max 40µl) of the crude enzyme extract. The increase in absorption at 340nm of 10 samples at 1 time was measured every 5 min for 1 h by the microplate reader utilising manufacturers software. Non-enzymatic conjugate formation was determined as described above using the scaled down method.

3.2.6. Relationship of soil sulphur supply and glutathione *S*-transferase activity.

Soil analysis was carried out to investigate the effect of macronutrient levels of sulphur (S) in the soil on GST activity in black-grass plants. Soil samples were collected from each site sampled using a trowel. This was opposed to using a soil corer, as the sites had routinely been ploughed and therefore sampling depth was not critical (Archer, 1988).

Ploughing resulted in the topsoil layer being well mixed and therefore macronutrients and OM were uniformly distributed throughout the soil. Up to 10 samples from 1 plot were taken and bulked together resulting in between 0.5 and 1.0 kg of soil in weight. Samples were taken by walking the plot in a W pattern and taking a soil sample every 1-2m. Care was taken to avoid the normal line of lime and fertiliser operations in the fields. These samples were analysed for organic matter, soil pH, magnesium, phosphorus, potassium and sulphur by ADAS Wolverhampton.

3.2.7. Relationship between climatic influences and GST activity in untreated black-grass plants harvested from the field.

Meteorological data, relating to each trial site, were obtained from stations nearest to each location, courtesy of the UK Meteorological Office, Bracknell, London. Data included maximum, minimum and mean air temperature, rainfall totals, relative humidity, sunshine hours and solar radiation data. This information was utilised to investigate the effects of climatic conditions on GST activity in the black-grass populations

3.2.8. Data analysis.

In all cases, data from one single site is presented, but is typical of all the sites studied. All the sites studied presented similar trends and correlations with respect to protein content and GST activity, although some anomalies were observed. Data presented in the following results was selected on the basis that the particular site shown illustrates the best correlation or trend for that complete set of data for every site studied.

Population counts for each site were expressed as means of untreated black-grass populations m^{-2} with standard errors also expressed using data collected during Year 2. Protein content and GST activity, comparing and contrasting between individual leaves at each growth stage sampled and between the growth stages themselves were based on

observations of 5 plants (Year 1) and 10 plants (Year 2). These values were expressed as protein content (mg g^{-1} fwt), GST specific activity (μmol CDNB min^{-1} mg^{-1} total protein) and GST activity (μmol CDNB g^{-1} fwt). Glutathione *S*-transferase activity is presented on both a protein (specific activity) and fresh weight basis. Determination of specific activity calculates the amount of GST activity per amount of total protein in the leaf or plant. Any changes in specific activity can therefore be attributed to there being more or less GST protein as a percentage of total protein. This suggests to some extent that specific synthesis of GST protein is increased or decreased in comparison to general protein or that GST protein has increased or decreased activity. When drawing conclusions, it is more appropriate to consider data expressed on a protein basis as it is more accurate to correlate GST activity to the protein content of each individual sample. GST activity that is calculated on a fresh weight basis determines the amount of activity g^{-1} fwt. This is an alternative method to specific activity determination and allows comparisons between GST activity and a plant weight situation to be examined. This method of GST determination takes into account factors other than protein synthesis, which may affect GST activity for example, the amount of water in the plant g^{-1} fwt. This method is simpler than calculating GST activity on a protein basis and goes some way to allowing results to be compared to those from the field. Both methods of determining GST activity are presented so as to explain whether GST activity at the cellular level (specific activity) is due to changes in GST protein or activity or if it may be due to other factors such as the plant being dehydrated (activity g^{-1} fwt).

Statistical analyses of these values were carried out using Genstat 5 Release 4.1. Linear regression was used to identify relationships in mean GST activity g^{-1} fresh weight between the individual leaves of each plant sampled of which a worked example and original data are presented in Appendix 2. In addition, T-tests were used to test the null hypothesis of zero mean difference at the 95% confidence level ($P<0.05$ = significant,

P>0.05 = non-significant) that protein content and endogenous GST activity within individual leaves did not increase as plants matured and developed from juvenility through to relative maturity. In all cases, standard errors were calculated.

Additional analysis was carried out if no relationships were exhibited between the individual leaves of each plant, as this suggested that data from the singles leaves of one plant could be averaged to indicate a whole plant response. This involved calculating the amount of GST activity within the individual leaves i.e. GST activity g^{-1} fwt multiplied by the individual leaf fresh weight. The values for the leaves of each individual plant were then added together and divided by the number of leaves on the plant, to give an average value for the plant as a whole together with standard errors. A similar calculation was carried out with respect to protein content. GST whole plant values were used for comparison with figures obtained courtesy of Syngenta Crop Protection UK Ltd of the effect of application timing on the control of black-grass by fenoxaprop-P-ethyl and clodinafop-propargyl. These observations were used to identify possible spray windows for farmers.

NB. The reader should note that if trying to compare and contrast GST data for individual leaves (Table 3.4) to that of whole plants (e.g. Figures 3.8 and 3.10), there may be no correlation due to the different calculations utilised to generate the data.

3.3 RESULTS

3.3.1. Plant growth and development.

Untreated black-grass plants sampled from all sites over the 2 year period were recorded for growth stage. Signs of leaf senescence as loss of photosynthetic pigments in older leaves were noted at Site 5 from November 1999 onwards and from January/February 1999/2000 at other sites. No other visible symptoms of foliar damage or injury were observed during any of the sampling periods.

3.3.2. Effect of crop and weed competition on untreated black-grass plants in the field.

Figure 3.2. illustrates the changing plant populations of black-grass at the 3 trial sites sampled between October 1999 and May 2000. Sites 3 and 4 had substantially lower infestations m^{-2} than site 5. Population counts at Site 5 were suspended from January 2000 due to the sheer number of plants m^{-2}. It was felt that further counts would be inaccurate due to problems identifying the base of individual plants. The difference in plant densities between Sites 4 and 5 are clearly illustrated in Figure 3.3. Visual assessment at all 3 sites after sampling had been concluded in the summer of 2000 indicated that the higher black-grass population at Site 5 had resulted in a lower number of wheat heads being produced than at Sites 3 and 4. However in contrast, at the lower weed densities assessed at Sites 3 and 4, more heads per black-grass plant were produced than on black-grass plants at Site 5. These observations are in agreement with those of Moss (1987a).

Crop and weed emergence at Site 5 coincided at the end of September/beginning of October 1999. Weed emergence at Sites 3 and 4 was observed to be much later in November 1999 as a "second flush" of emerging black-grass. Visually, the effect of black-grass growth on crop growth in terms of competition at Sites 3 and 4 was relatively small

prior to April 2000. There was a small visually detectable effect on crop growth at Site 5 early in the growing season due to the presence of a significantly larger black-grass population m^{-2}. At the March 2000 sampling point the black-grass and cereals were at the same growth stages of GS30/31. However, at the final sampling date in May 2000, the black-grass populations had over taken the crops in terms of growth stage in concurrence with the observations of Moss (1987a).

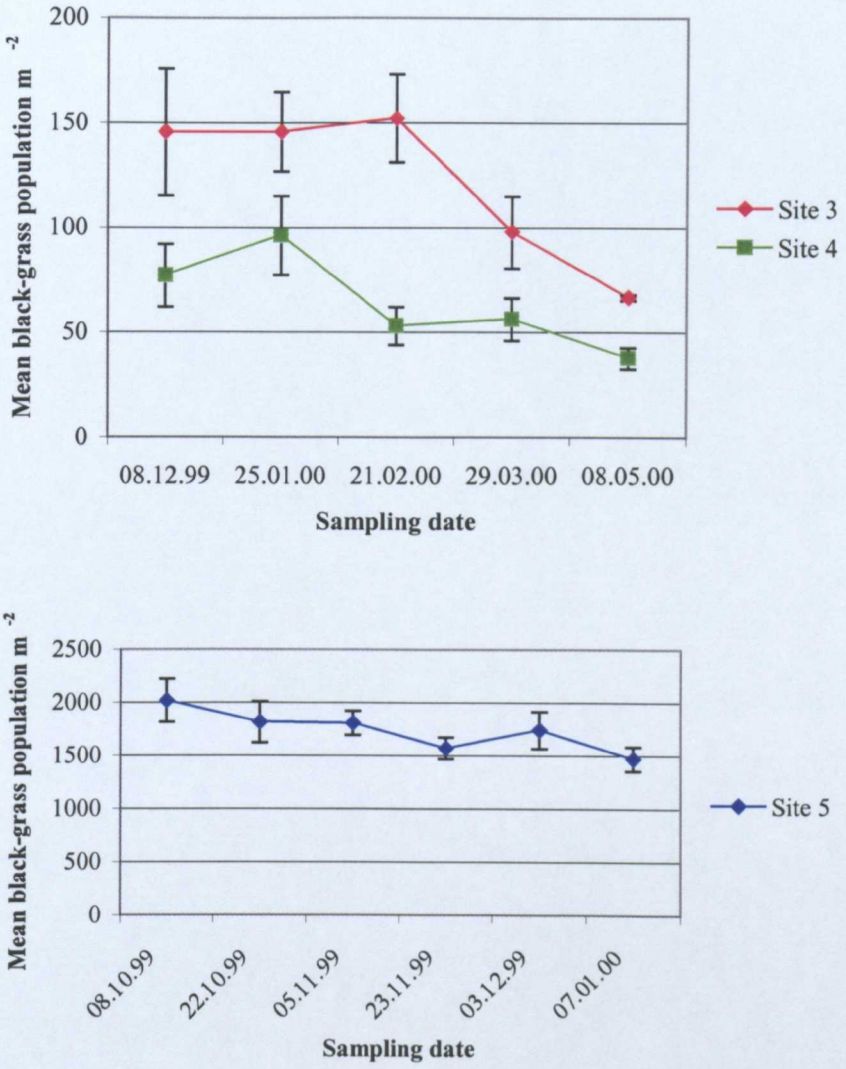


Figure 3.2. Mean untreated black-grass populations m^{-2} at Sites 3, 4 and 5. Plants sampled at different growth stages between October 1999 and May 2000. NOTE: Population assessments at Site 5 ceased after January 2000 due to the size of the population and difficulty in obtaining accurate counts. Population values are means ±SE values, where n = an average of 10 random quadrat counts.

Site 4 Average GS23

Site 5 Average GS29

Figure 3.3. Contrasting black-grass plant population density at Sites 4 and 5. Photographs taken 21st February 2000.

3.3.3. Protein content in untreated black-grass leaves sampled from 5 differing cereal field-trial sites over 2 consecutive years.

Protein content on a fresh weight basis was relatively low during December/January of both years. Mean soluble protein in individual black-grass leaves at each site was shown to increase significantly ($P<0.05$) at different developmental stages as plants matured, as shown in Figure 3.4. Additional data exhibiting mean soluble protein content from Sites 1 and 3 –5 are available on request. The magnitude of the responses was dependent on individual leaf age, with younger leaves having significantly more ($P<0.05$) protein. As plants matured, significant reductions ($P<0.05$) in soluble protein content were observed in older, senesced leaves at the final sampling points at each site. Statistical analyses by means of t-tests testing the hypothesis of zero mean difference were carried out to assess the changes in protein content over time in individual leaves. A summary of the protein results for each site is presented in Table 3.2.

Table 3.2. Summary table of significant observations with respect to mean soluble protein content (mg g^{-1} fwt) of individual black-grass leaves.

Observed parameters	SITE				
	1	2	3	4	5
↑ Protein with time	√	√	X	√	√
↓ Protein in winter	√	√	X	√	√
↑ Protein in spring	√	√	X	√	√
Age effect	√	√	√	√	√

Key: ↑ - increased, ↓ - decreased, √ - Yes, X - No

Site 3 did not follow the trend exhibited by the other four sites as illustrated in Table 3.2. This may have been due to a number of reasons such as soil pH and OM content, soil sulphur, the particular season or environmental conditions and are discussed later in further detail.

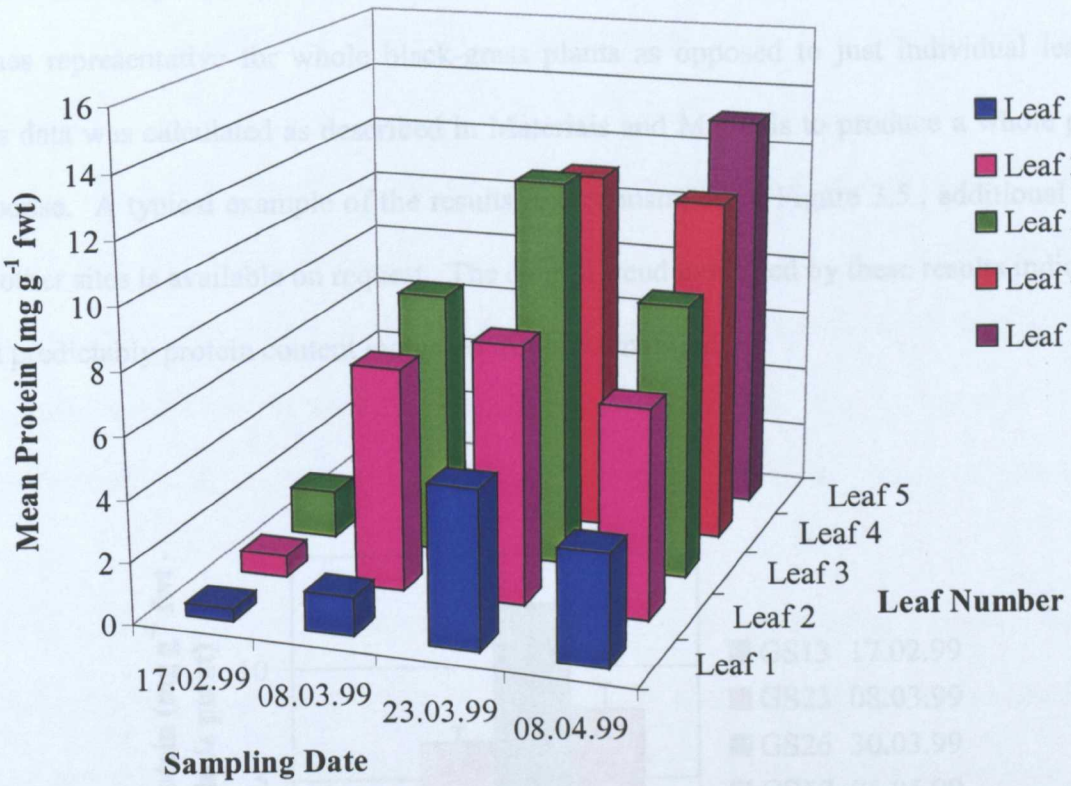


Figure 3.4. Mean soluble protein content (mg g^{-1} fwt) for individual black-grass leaves of differing maturity, harvested from Site 2. Protein content values are means, where $n = 5$ leaves. Leaf 1 corresponds to the first fully emerged leaf. Subsequent leaves were numbered sequentially. As growth stages advanced, more leaves were available for analysis. These data represent one single site but are typical of all the sites studied.

Protein content of Site 2 was observed to significantly increase ($P<0.05$) in individual leaves over time and significantly decreased ($P<0.01$) at the last sampling date in leaf 3 and non-significantly in leaves 1 and 3 as illustrated in Figure 3.4. The observation of higher protein content in younger leaves was clearly defined as can be seen in Figure 3.4. and in some cases, this was significant ($P<0.05$). Decreases in protein content at the final sampling point were observed in every leaf as illustrated in Figure 3.4. with the exception of leaf 5. This decrease was only significant ($P<0.01$) in leaf 3.

Additional analysis of protein content was carried out in an attempt to produce protein values representative for whole black-grass plants as opposed to just individual leaves. This data was calculated as described in Materials and Methods to produce a whole plant response. A typical example of the results is demonstrated in Figure 3.5., additional data for other sites is available on request. The overall trend exhibited by these results indicated that predictably protein content increased as plants matured.

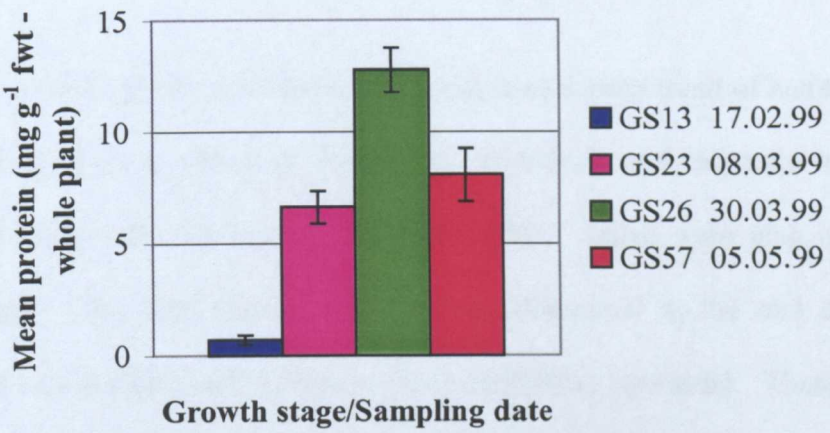


Figure 3.5. Mean soluble whole plant protein (mg g^{-1} fwt – whole plant) for black-grass plants of differing maturity, harvested from Site 1. Protein content values are means ±SE values, where n = 5 whole plants. These data represent 1 single site but are typical of all the sites studied.

Whole plant protein content of plants harvested from Site 1 significantly increased ($P<0.001$) until the end of March 1999 as demonstrated in Figure 3.5. after which it significantly decreased ($P<0.05$) with maturation and flag leaf development. This data was comparable with the individual leaf data for this site.

3.3.4. Determination of glutathione *S*-transferase specific activity in individual black-grass leaves harvested at different growth stages from 5 differing black-grass populations.

GST specific activity within individual black-grass leaves was shown to naturally elevate as black-grass plants developed over the winter to spring period, as illustrated in Figure 3.6. Statistical analysis by means of linear regression was carried out to determine any relationships between leaves at each sampling point. In addition, t-tests were used to investigate any differences in activity of individual leaves at different stages of maturity.

Observations of GST specific activity at site 3 indicated a clear trend of increasing activity at each sampling point as shown in Figure 3.6. However, none of these increases were significant ($P>0.05$) until the end of March ($P<0.05$). There were also indications, as shown in Figure 3.6., that specific GST activity decreased at the end of tillering in maturing plants as the flag leaf became visible (GS39/May onwards). These decreases in activity, particularly for leaves 1,3, and 4 were significant ($P<0.05$). Linear regression analysis indicated that there was no relationship between the different individual leaves and GST specific activity until February and March ($P<0.05$). However, at the final sampling point in May, the relationship was again non-significant ($P>0.05$). Data from the remaining sites are available on request. Table 3.3. presents an overall summary of significant observations relating to GST specific activity at every site.

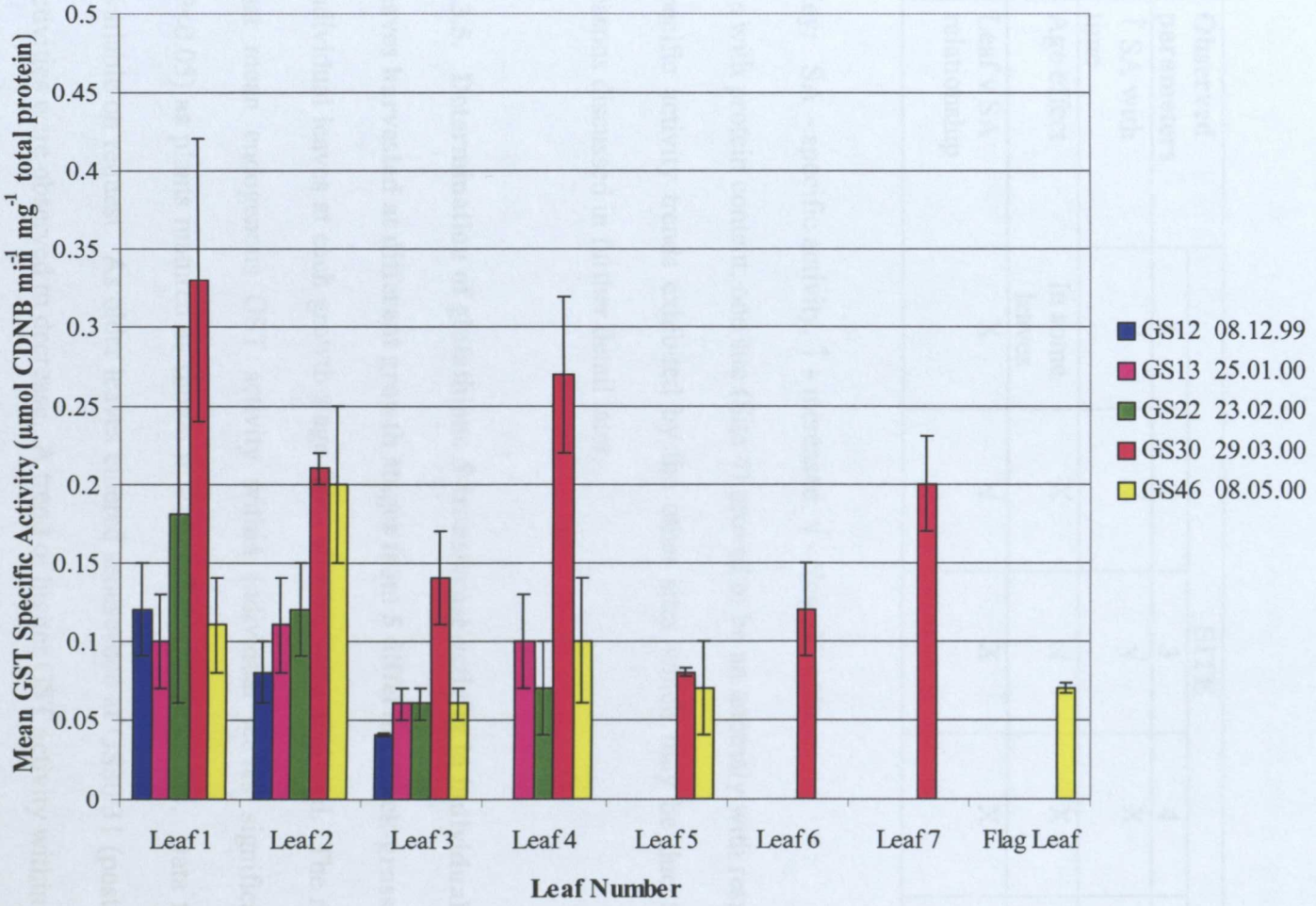


Figure 3.6. Mean GST specific activity (µmol CDNB min^{-1} mg^{-1} total protein) of individual black-grass leaves harvested from Site 3. Leaf values are means ±SE values, where n = 10 leaves. Leaf 1 corresponds to the first fully emerged leaf. Subsequent leaves were numbered sequentially. As growth stages advanced, more leaves were available for analysis. These data represent 1 single site but are typical of all the sites studied.

Table 3.3. Summary table of overall significant observations with respect to mean GST specific activity (μmol CDNB min^{-1} mg^{-1} total protein) of individual black-grass leaves.

Observed parameters	SITE				
	1	2	3	4	5
↑ SA with time	√	√	√	X	√
Age effect	In some leaves	X	√	X	√
Leaf v SA relationship	X	√	X	X	Some Significant (P<0.001)

Key: SA – specific activity, ↑ - increased, √ - Yes, X – No

As with protein content, one site (Site 4) proved to be an anomaly with respect to the GST specific activity trends exhibited by the other sites which may be due to a number of reasons discussed in further detail later.

3.3.5. Determination of glutathione *S*-transferase activity in individual black-grass leaves harvested at different growth stages from 5 differing black-grass populations.

Individual leaves at each growth stage from all sites were analysed. The results indicated that mean endogenous GST activity within individual leaves significantly increased ($P<0.05$) as plants matured as shown in Figure 3.7. and Table 3.4. Data from other sites available on request. As older leaves entered senescence at GS30/31 (post-tillering), GST activities were observed to decrease. A trend of higher GST activity within younger leaves was also prevalent as was demonstrated with protein content.

The results for site 2 are presented in Figure 3.7. As plants matured, increases in GST activity were observed at every growth stage. Some of these increases were significant ($P<0.05$). At the second sampling point, GST activity within every leaf increased by over

600% of that at the initial sampling point. Increases in activity were observed at every growth stage as illustrated by Figure 3.7, but they became progressively lower increases as plants matured. Linear regression analysis indicated that there was no significant difference ($P>0.05$) in mean GST activity between the individual leaves of each plant sampled at any growth stage.

Statistical analysis through linear regression indicated that at each sampling growth stage, there was no significant difference ($P>0.05$) in mean GST activity between the individual leaves of each plant sampled (up to GS30/31). This suggests that data from single leaves of 1 plant can be averaged to indicate a whole plant response, as illustrated in Figure 3.8. Whole plant GST values were used for comparison with historical data obtained by Syngenta Crop Protection UK Ltd for the effect of application timing on the control of black-grass by fenoxaprop-P-ethyl from several commercial field trials. The results indicate a trend in which herbicide efficacy declined from February onwards, whereas GST activity increased. GST activity was lowest during December and January, when efficacy was high. GST activity peaked at either the end of April or the beginning of May, at growth stage 30/31 at each site as illustrated in Figure 3.8. It is speculated that this data may be utilised to predict spray windows with respect to obtaining maximum herbicide efficacy. However, it must be acknowledged that weed and crop growth stage, the particular season and environmental conditions are also used to identify windows, all of which can also contribute to reduced herbicide efficacy.

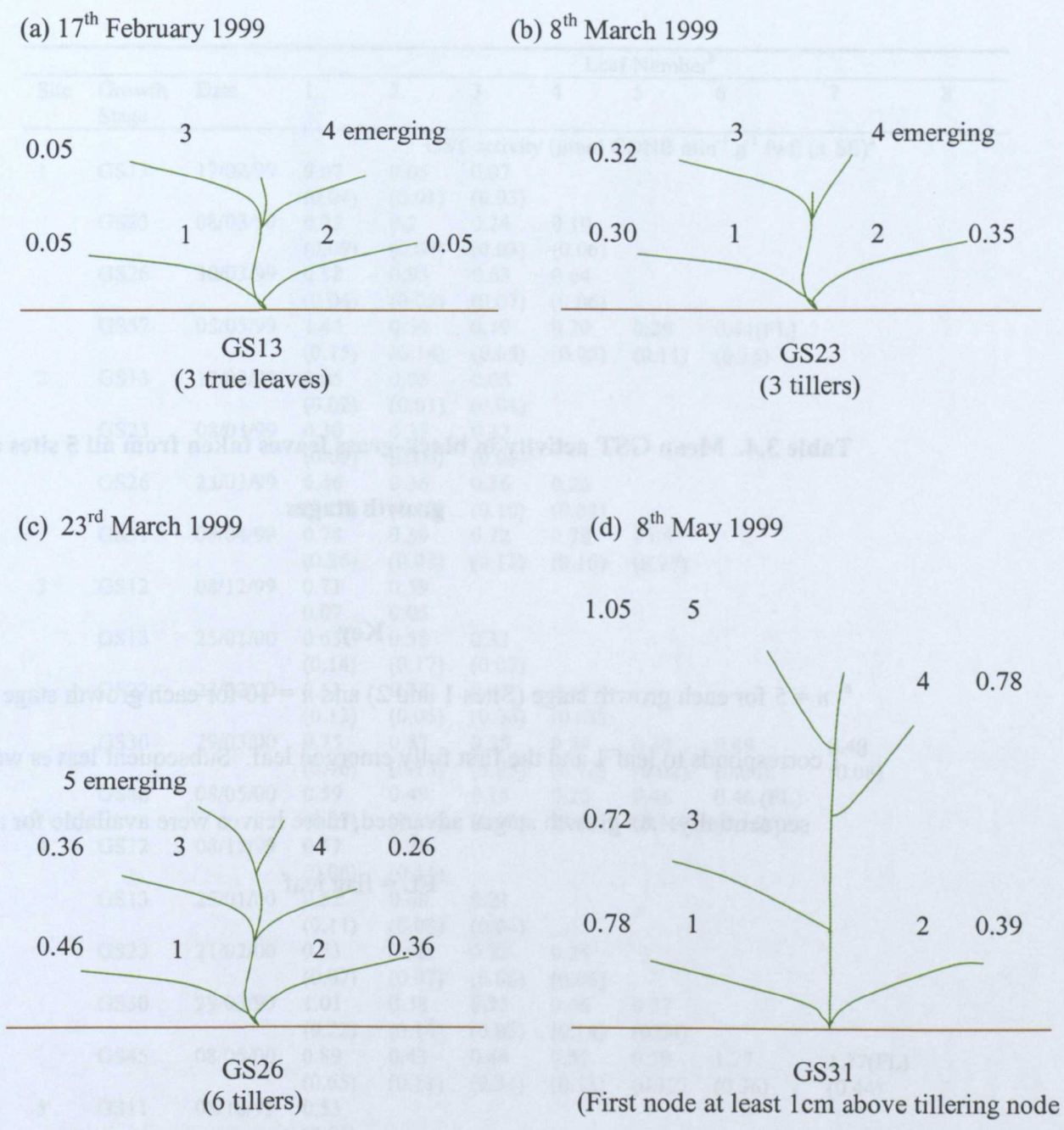


Figure 3.7. Mean GST activity (µmol CDNB min^{-1} g^{-1} fwt) in black-grass leaves taken from Site 2 at 4 growth stages. Values are means (n = 5 leaves) for each growth stage. Leaf 1 corresponds to the first fully expanded leaf. Subsequent leaves were numbered sequentially. As growth stages advanced, more leaves were available for analysis. These data represent 1 single site but are typical of all sites studied.

Please see overleaf of this page for key referring to Table 3.4.

Table 3.4. Mean GST activity in black-grass leaves taken from all 5 sites at different growth stages.

Key

[a] $n = 5$ for each growth stage (Sites 1 and 2) and $n = 10$ for each growth stage (Sites 3 –5)

[b] 1 corresponds to leaf 1 and the first fully emerged leaf. Subsequent leaves were numbered sequentially. As growth stages advanced, more leaves were available for analysis.

FL = flag leaf

Site	Growth Stage	Date	Leaf Number[b] 1	2	3	4	5	6	7	8
			GST activity (μmol CDNB min^{-1} g^{-1} fwt) (± SE)[a]							
1	GS13	17/02/99	0.07 (0.04)	0.05 (0.01)	0.07 (0.03)					
	GS23	08/03/99	0.23 (0.09)	0.2 (0.04)	0.24 (0.03)	0.19 (0.06)				
	GS26	30/03/99	0.58 (0.04)	0.93 (0.05)	0.63 (0.07)	0.64 (0.06)				
	GS57	05/05/99	1.44 (0.15)	0.54 (0.14)	0.19 (0.05)	0.20 (0.05)	0.29 (0.11)	0.41(FL) (0.15)		
2	GS13	17/02/99	0.05 (0.02)	0.05 (0.01)	0.05 (0.01)					
	GS23	08/03/99	0.30 (0.09)	0.35 (0.04)	0.32 (0.06)					
	GS26	23/03/99	0.46 (0.20)	0.36 (0.10)	0.36 (0.10)	0.26 (0.03)				
	GS31	08/04/99	0.78 (0.36)	0.39 (0.08)	0.72 (0.13)	0.78 (0.16)	1.05 (0.27)			
3	GS12	08/12/99	0.71 0.07	0.59 0.05						
	GS13	25/01/00	0.63 (0.14)	0.58 (0.17)	0.33 (0.07)					
	GS22	23/02/00	0.61 (0.13)	0.37 (0.06)	0.37 (0.06)	0.47 (0.08)				
	GS30	29/03/00	0.75 (0.16)	0.87 (0.13)	0.35 (0.05)	0.54 (0.12)	0.30 (0.02)	0.49 (0.06)	0.40 (0.08)	
	GS46	08/05/00	0.59 (0.07)	0.49 (0.10)	0.15 (0.05)	0.20 (0.03)	0.46 (0.16)	0.46 (FL) (0.16)		
4	GS12	08/12/99	0.57 (0.06)	0.38 (0.11)						
	GS13	25/01/00	0.52 (0.11)	0.48 (0.08)	0.21 (0.04)					
	GS23	21/02/00	0.53 (0.07)	0.38 (0.07)	0.32 (0.06)	0.25 (0.06)				
	GS30	29/03/99	1.01 (0.22)	0.38 (0.14)	0.21 (0.05)	0.46 (0.14)	0.37 (0.04)			
	GS45	08/05/00	0.89 (0.65)	0.43 (0.11)	0.44 (0.34)	0.51 (0.13)	0.59 (0.12)	1.77 (0.36)	1.77(FL) (0.44)	
5	GS11	08/10/99	0.53 (0.05)							
	GS13	22/10/99	0.48 (0.06)	0.41 (0.03)	0.34 (0.05)					
	GS22	05/11/99	0.76 (0.19)	0.56 (0.17)	0.38 (0.07)	0.32 (0.03)				
	GS23	23/11/99	0.32 (0.04)	0.30 (0.02)	0.39 (0.05)	0.41 (0.08)				
	GS24	03/12/99	0.56 (0.09)	0.32 (0.07)	0.29 (0.03)	0.30 (0.03)	0.35 (0.1)			
	GS26	07/01/00	0.41 (0.12)	0.17 (0.01)	0.19 (0.03)	0.36 (0.03)	0.21 (0.03)			
	GS28	25/01/00	0.52 (0.14)	0.17 (0.05)	0.20 (0.04)	0.34 (0.03)				
	GS29	21/02/00	0.45 (0.08)	0.52 (0.12)	0.38 (0.07)	0.48 (0.10)	0.44 (0.04)			
	GS31	29/03/99	0.59 0.10)	1.42 (0.24)	0.85 (0.21)	0.26 (0.13)	0.22 (0.12)	0.27 (0.17)	0.17 (0.02)	0.16 (0.04)
	GS48	08/05/00	0.67 (0.23)	0.60 (0.11)	0.22 (0.06)	0.27 (0.05)	0.28 (0.03)	0.53 (0.12)	0.60(FL) (0.22)	

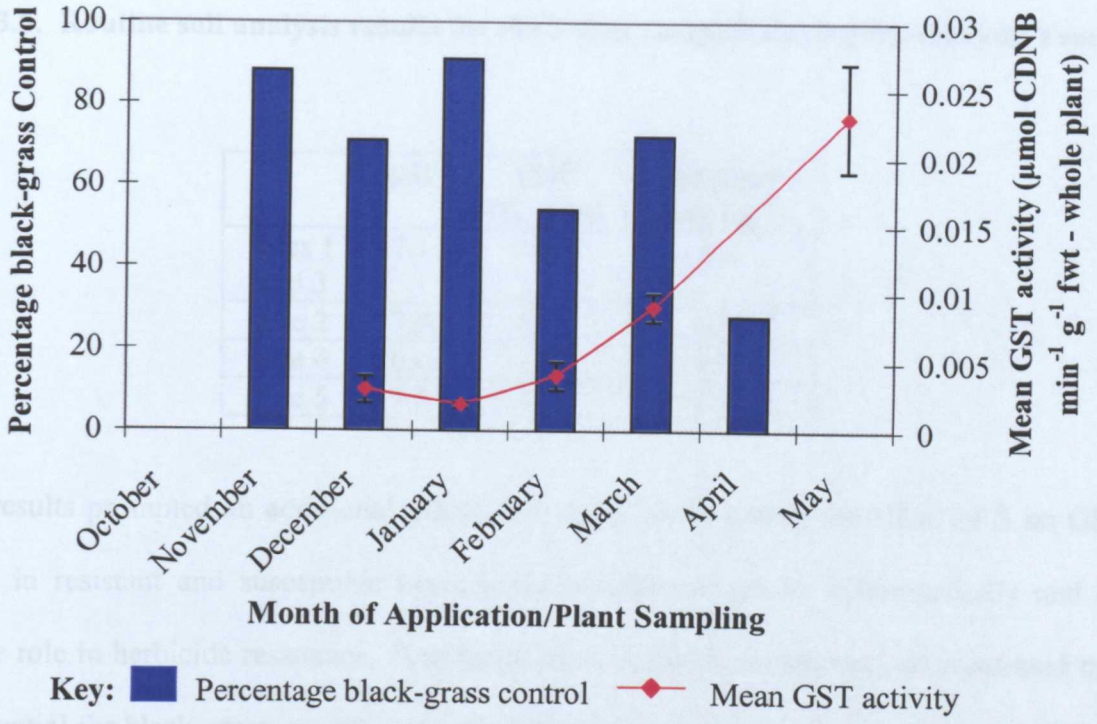


Figure 3.8. Effect of application timing on the control of resistant black-grass populations by fenoxaprop-P-ethyl. Historical data taken from several commercial field trials. Additional data on mean GST activity from Site 4 are overlaid for comparison. GST values are means ±SE values, where n = 10 plants.

3.3.6. Relationship between soil sulphur content and GST activity.

Soil analyses from each site (Table 3.5.) indicate that the 5 sites were relatively similar in terms of soil pH and OM content. The critical concentration of S required within soil to prevent deficiency symptoms in cereals is 10-12 mg kg^{-1} (Scott, 1981). Thus sites 1 and 3 were severely deficient in S and site 5 had only the lowest recommended critical level. In contrast, sites 2 and 4 had sufficient S. These observations could imply sites 1 and 3 may experience large cereal yield losses (MAFF, 1994).

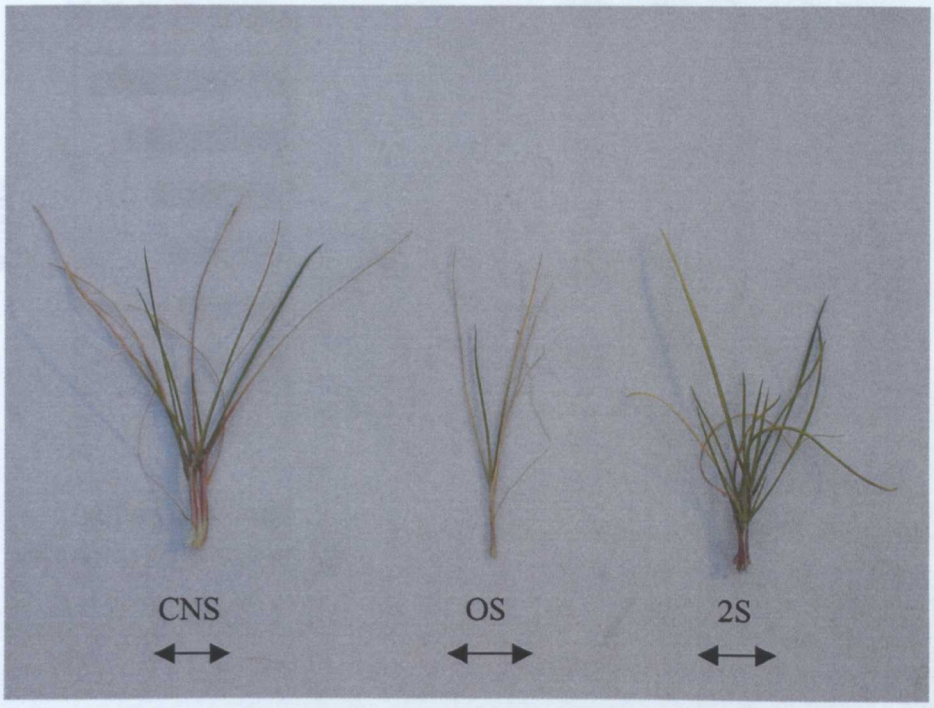
Table 3.5. Routine soil analysis results for the 5 sites sampled during the study in Year 2.

	pH	OM (% m/m)	Sulphur (mg kg^{-1})
Sites 1 and 3	7.1	4.11	6.3
Site 2	7.9	4.48	19.5
Site 4	6.0	4.48	20.0
Site 5	7.5	4.39	10.2

These results prompted an additional glasshouse study investigating the effect of S on GST activity in resistant and susceptible black-grass populations grown hydroponically and its possible role in herbicide resistance. The study (data available on request) demonstrated that S is essential for black-grass growth and development and plants were particularly sensitive to S deficiency as illustrated in Figure 3.9. The differences in GST activity between resistant and susceptible black-grass populations were substantiated irrespective of nutrient deficiency. There were no indications from this preliminary experiment that S status in the growth medium was proportional to GST activity, hence any possible link to herbicide resistance remains tenuous.

3.3.7. Relationship between climatic influences and GST activity in untreated black-grass plants harvested from the field.

No meteorological anomalies during the 2 growing seasons were observed from climate data relating to each trial site. However, the absence of stressful conditions cannot rule out the theory that the observed changes in GST activity could be attributed to natural fluctuations in climatic factors. Clear correlations between GST activity and mean monthly temperature, solar radiation and sunshine hours were observed, as shown in Figure 3.10. No correlation was observed between GST activity, rainfall and relative humidity.

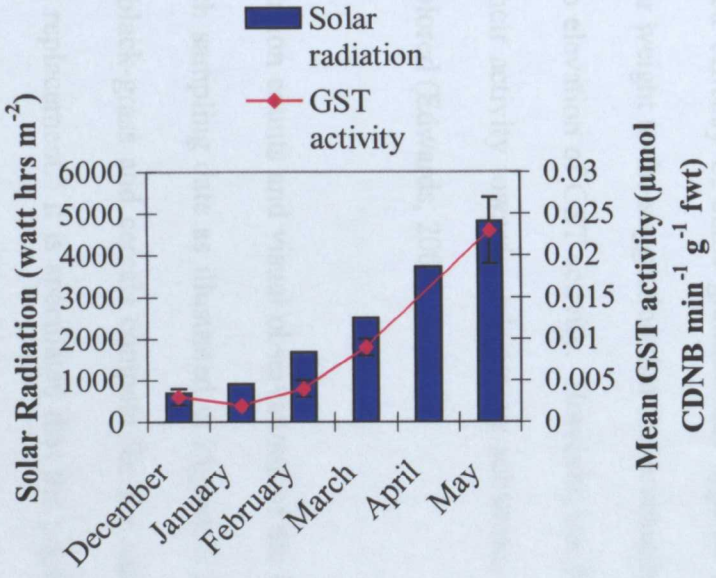


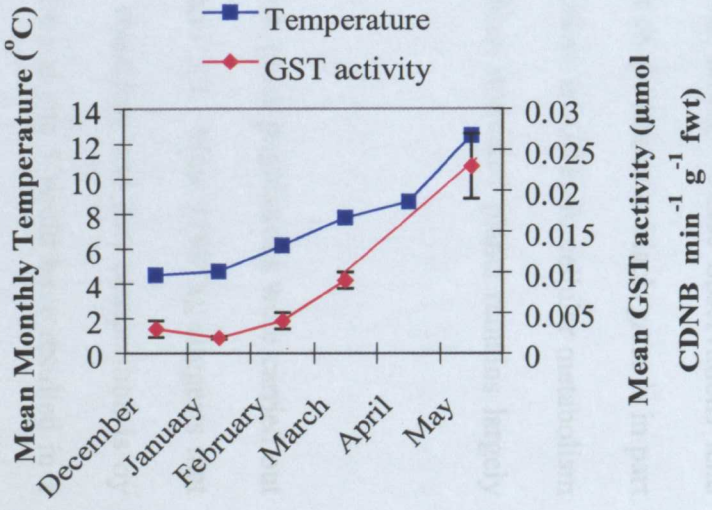
Key: **CNS** – Optimum nutrient solution; **OS** – Zero sulphur solution;

2S – 2X optimum sulphur

◄——► = 1cm

Figure 3.9. The effect of different sulphur concentrations on the growth of the black-grass population Herbiseed. Photograph taken 64 days post germination.





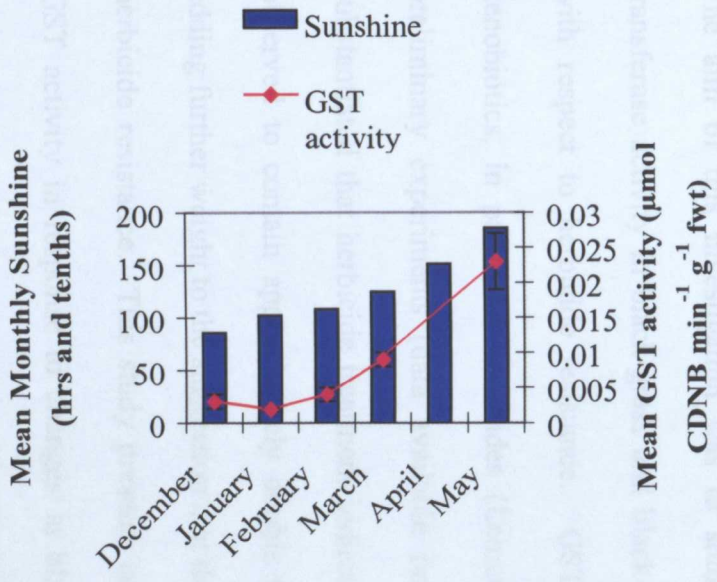


Figure 3.10. Mean monthly solar radiation, mean monthly sunshine and mean monthly temperature for Site 4. Additional data on mean GST activity are overlaid for comparison. GST values are means ±SE values, where n = 10 plants.

3.4. DISCUSSION

The aim of this investigation was to study the relationship between glutathione *S*-transferase activity in black-grass and black-grass growth and development in the field with respect to herbicide resistance. GSTs play key roles in the detoxification of xenobiotics, in particular herbicides (Lamoureux and Rusness, 1989, 1993). Indeed, preliminary experiments (data available on request) on Herbiseed and Peldon have substantiated that herbicide treatment induces GST activity in black-grass. Peldon was observed to contain approximately double the GST activity of susceptible Herbiseed, adding further weight to the suggestion that there is a correlation between GST activity and herbicide resistance. This study presents novel observations of increasing endogenous GST activity in response to changes in black-grass growth and development and the seasonal variation in environmental conditions. All plants sampled showed similar trends with respect to GST activity and protein content with age. It is speculated that this endogenous change in enzyme activity with plant development in the field contributes to reduced efficacy of some graminicides applied in the spring. These observations lend further weight to the suggestion that the development of resistance in black-grass is in part due to elevation of GST activity. However, the functions of GSTs in cellular metabolism and their activity towards endogenous substrates within untreated plants remains largely unexplored (Edwards, 2000).

Population counts and visual observations of the black-grass populations were carried out at each sampling date as illustrated in Figures 3.2. and 3.3. Moss (1987a), suggests that both black-grass and cereals compete for the same resources and that competition is by direct replacement. It is speculated that the population at site 5 could have resulted in a substantial yield loss. The population recorded was up to 4 times greater than the densities of 250-500 plants m^{-2} proposed of being capable of reducing yield by approximately 45%

(Moss, 1987a). In contrast, the population densities recorded at the other sites would probably have had little effect on crop yield with densities below 150 and 100 plants m^{-2}, respectively (Figure 3.4).

Soluble protein content in individual black-grass leaves and whole plants significantly increased ($P<0.05$) as plants matured (Figures 3.4. and 3.5.) with the exception of site 3. The results suggest protein content was dependent upon leaf age, i.e. the younger the leaf, the more protein present, which corresponds to the observations of Schwanz and Polle (1998). This is further confirmed by losses in protein content observed from March onwards in both years at all sites, predominantly in older leaves. Leaf protein content at site 5, significantly increased ($P<0.05$) until December 1999. Protein loss within individual leaves was then observed in correspondence with the other sites and the onset of winter. Decreases in protein content at this time may be attributed to the dormant growth stage exhibited by plants in response to the changing season, i.e. winter to spring. Grasses are known to pass through a phase of lower metabolic activity at the onset of winter (Langer, 1979; Simon, Dormer and Hartshorne, 1980). Protein content peaked at GS30/31 at every site, i.e. end of tillering and moving into the stem elongation stage (Figure 3.4.) which corresponded with the end of April/beginning of May in both years.

Increasing endogenous GST specific activity (Figure 3.6. and Table 3.3) was also substantiated with black-grass growth and development and changing season, but with fluctuations evident which is in contrast to the glasshouse observations of Cummins *et al.*, (1997a). However, this study was carried out over a longer period, using plants from the field, with longer breaks between sampling. Many of the increases exhibited were not significant ($P>0.05$), but trends were clear in the data. Similar to protein content, specific activity was highest in young leaves and seen to culminate at the end of March (GS30/31) followed by a decline.

GST activity g^{-1} fwt (Figure 3.7. and Table 3.4.) significantly ($P < 0.05$) elevated every growth stage at each site and fluctuated with changing season and black-grass growth. This corresponds to the observations of Badiani, Paolacci, Miglietta, Kimball, Pinter, Garcia, Hunsaker, LaMorte and Wall, (1996) with respect to antioxidants in wheat leaves which fluctuated in a cyclic, non-regular manner through changing seasons. As for protein content, whole plant data for GST activity were calculated illustrating significant ($P<0.05$) fluctuations in GST activity with changing season and black-grass development.

Soil analysis (Table 3.3) indicated sites 1 and 3 were severely deficient in S, which may have led to the results exhibited by site 3 not being so conclusive. In addition, site 5 soil bordered on S deficiency. A preliminary glasshouse experiment investigating the effect of S on GST activity in resistant and susceptible black-grass revealed that S is essential for black-grass growth and development, but a possible role in herbicide resistance remains to be verified. Clear correlations between GST activity, temperature, solar radiation and sunshine hours were observed (Figure 3.10.) adding further weight to the suggestion that these natural changes in GST activity could be attributed to natural fluctuations in climatic factors.

The 5 sites studied exhibited similar trends and relationships, with a degree of synchronisation with respect to growth stage and changing season. However, as expected, there was some variation between the results with Site 3 proving to be an anomaly with respect to protein content and Site 4 with respect to GST specific activity, demonstrating different trends and results. This may have been as a result of the pH of the soil, soil S and OM content, the particular season or environmental conditions. The evidence of S deficiency at Site 3 and the results of the preliminary glasshouse study indicate that this area is worthy of further study.

This investigation was labour intensive with respect to the travelling required to the sites to sample plants and the time taken to document, dissect and analyse plant material on arrival at the laboratory. This meant that only untreated plants were sampled. The investigation would have benefited from plots being included that had been treated with different herbicides in order to carry out a comparison against untreated plants. This could have further confirmed the role herbicides play in inducing GST activity in black-grass and would have been a good comparison to GST activity measured in untreated plants over time. However, time and space limitations prevented this. Another valuable comparison would have been to include a standard susceptible population alongside the plots. However, it would have been impossible to identify a completely susceptible field black-grass situation as most populations are made up of both resistant and susceptible individuals. In hindsight, growing a susceptible standard such as Herbiseed in a glasshouse may have sufficed, but extrapolating the results to the field for comparison would have been difficult due to very different growth conditions.

The study would also have benefited from the inclusion of additional sites located in a wider geographical area so that influencing factors such as location, soil type and environment could have been more widely studied. Sampling size was also a limiting factor, although it was increased during year 2 to further validate the data. A larger sampling size would have been beneficial, but was not possible due to time restraints with respect to laboratory analysis, particularly as plants developed and produced more leaves. This also led to time between sampling dates being increased from every 2 weeks initially to 1 month, but this coincided with the rate of plant growth and development decreasing to an extent.

The progressive increases observed in GST activity (Figures 3.6 – 3.8.) may have been the result of the sampling protocol developed and/or the analytical procedures employed post

sampling. However, only fully expanded leaves were harvested but at later growth stages, older leaves were senescent and may have been physiologically different. Leaf position on the main stem may also have caused variations in GST activity. Although individual leaves were assayed during this study, no attempt was made to assess activity in the roots of black-grass plants. It would have been difficult to remove the plants from the soil and guarantee that the roots attached were intact. In addition, complete removal of the surrounding soil would have been difficult leading to contaminated samples. Variations between samples harvested may also have been caused by unavoidable fluctuations in field conditions, coupled with diurnal changes in metabolic activity in the plants (Foyer, 1993; Hausladen and Alscher, 1993; Badiani, Schenone, Paolacci and Fumagalli, 1994). It is speculated that these variations were minimised in this study as plants were always sampled from the same field locations at approximately the same time of day. The surrounding conditions of the black-grass plants could have resulted in variations in growth *in situ* and therefore enzyme activity. Immediate and long-term storage of samples and the analytical conditions they were exposed to were unlikely to have resulted in the fluctuating patterns observed for changing season. Plant material was documented and dissected upon immediate arrival back at the laboratory and stored at -80°C until further analysis could take place. No inadvertent thawing during storage was observed. Protein and GST extraction and analysis was carried out under standardised and reproducible conditions and established assay methods were followed.

The soil type at sites 1 and 3 was documented as clay whilst soils which are most likely to show S deficiency in England and Wales are sandy, free draining and alkaline (Archer, 1988). The soil analysis did not take into account subsoil nutrients. Therefore, if sufficient subsoil reserves of S were present at sites 1 and 3, the deficiency may have been balanced out. Any conclusions drawn here therefore must be treated with caution and explored through further study.

It is well documented that many physiological and metabolic strategies are adopted by plants to adapt to natural fluctuations in their environment (McKersie and Lesham, 1994; Badiani, Paolacci, Fusari, D'Ovidio, Scandalios, Porceddu and Sermanni, 1997). Variations in GST activity between plant species are known to occur naturally (Marrs, 1996), but few studies have been carried out to assess the role of GSTs between differing populations within the same species. Consequently they could provide an intrinsic detoxification protection mechanism conferring herbicide resistance (Sharples *et al.*, 1995; Reade *et al.*, 1997). The progressive increase in enzyme activity reported in Figures 3.7. and 3.8. may simply reflect the development of black-grass leaves and whole plants, i.e. juvenility to senescence, and their response to natural fluctuations in climatic factors (Figure 3.10.), as shown in wheat leaves by Badiani *et al.*, (1996). Analysis of climatic data relating to each site gave no evidence to suggest that these natural elevations in activity were due to stress induced by abiotic, biotic or environmental/climatic factors. However, the absence of stressful conditions cannot rule out the theory that natural fluctuations in climatic factors attributed to the observed changes in GST activity as a response to oxidative stress induced by changing climatic conditions.

Both GST activity and protein content were observed to peak at GS30/31, (Figures 3.4., 3.6. and 3.7). As young, rapidly growing plants mature, metabolism is accelerated and a higher rate of reactive oxygen species (AOS) accompanies this (Gressel and Galun, 1994). Protection within the plant against endogenous and transient oxidative stress associated with plant developmental stages comes from increases in antioxidant enzyme abundance and activity (Badiani *et al.*, 1996). It is well documented that foliar protein and antioxidant enzymes show seasonal changes, developmental fluctuations and stress responses in both perennial and annual plants and tree species (Gillham and Dodge, 1987; Anderson, Chevone and Hess, 1992; Hausladen and Alscher, 1993; Kröniger, Rennenberg and Polle, 1993; Polle and Rennenberg, 1994). Studies have also shown that the amount of

antioxidant enzymes available for extraction varies with the physiological age of leaves (Foyer, 1993; Pastori and del Rio, 1994). However, it is only recently that there has been suggestion that developmental control plays a role in these fluctuations (Kröniger *et al.*, 1993; Badiani *et al.*, 1996) as speculated from this study. It is postulated that GSTs form part of this protection mechanism and that increasing endogenous GST activity is required as part of a plants natural defence system during vegetative growth. This is in order to protect developing plants from toxic endogenous compounds associated with changing growth development. It is conceivable that, during these essential phases of growth, GST activity is altered to keep pace with higher growth rates or the transition from vegetative through to reproductive development. This suggests that GSTs have direct cytoprotective activity and protect the plant during environmental changes and stress in conjunction with supporting normal plant development. In agreement with Cummins *et al.*, (1997) it is speculated that, although high GST activity was expressed in the populations observed in this study, it was unlikely that these GSTs were unique to these populations. Therefore, they were more likely to represent GST subunits, which are not expressed constitutively in untreated populations.

Soil sampled from sites 1 and 3 was found to be severely deficient in S. The roots and shoots of several plant species are supplied with reduced S in the form of GSH via long-distance transport, which is required for protein synthesis of these developing tissues (Rennenberg and Lamoureux, 1990). It is well documented that the amount of soil S present affects the concentration status of GSH in plants (De Kok, Maas, Godeke, Haaksma and Kuiper, 1986; Macnicol and Randall, 1987). Direct GSH conjugation reactions can occur in biological systems, but more often than not, they are generally catalysed by GSTs (Clarke, Greenhow and Adams, 1998). It is well documented that varying GSH content can affect GST activity (Scarponi, Perucci and Marinetti, 1991; Lamoureux and Rusness, 1993). Under S starvation conditions, the intracellular GSH pool

of tobacco cells declines (Smith, 1975). It is therefore postulated that soil S may indirectly have affected GST activity in untreated black-grass plants through depleted GSH content. However, as leaf GSH concentration was not assessed, it is not possible to ascertain how much was present in the individual black-grass leaves and thus what effects it may have had on GST activity. The glutathione pool size was not measured in this study as the crude protein extracts were desalted through Sephadex PD10 columns and GSH, being a small molecule, was discarded during this process. Any conclusions drawn here therefore must be treated with caution and explored by repetition and further study.

The deleterious effects of black-grass populations on cereal yields and the relative earliness in the growing season with which it becomes established, dictates that is should be controlled in the autumn if maximum yield potential is to be realised. Incidence of decreased herbicide efficacy within both herbicide-resistant and –susceptible maturing black-grass populations, effects of resistance and environmental factors are well-documented (Blair, Clarke and Orson, 1996). The role of GSTs in determining herbicide tolerance and thus selectivity through detoxification within crops and weeds is well-established (Hatton *et al.*, 1996; Marrs, 1996). The increase in innate GST activities shown here may explain the overall trend of reduced herbicide efficacy observed from February onwards in the field (Figure 3.11). The results from this study, together with observations from Syngenta Crop Protection UK Ltd (Mills and Ryan, 1995; Ryan and Mills, 1997), indicate that the timing of herbicide application is critical, especially where resistant black-grass populations are suspected.

Hatton *et al.*, (1996) suggested that herbicide selectivity could be predicted in weed species from their respective GST activities with a range of herbicide substrates. Their studies revealed a link between GST enzyme activity and rates of GSH conjugation *in vivo* and relative rates of detoxification and sensitivity to herbicides. GST activity or abundance can

be correlated with resistance of a black-grass population to fenoxaprop-P-ethyl and this method can be used to assess herbicide resistance in a population (Reade *et al.*, 1999; Reade and Cobb, 2002). Predictions of the timing of herbicide applications in black-grass utilising GST activities could be carried out based on the findings of this study. The results indicate that applying herbicides at times when GST activity is relatively low would lead to maximum efficacy. It is speculated that applications would be most effective early in the autumn when plants germinate, and during January, if a second flush of black-grass appears in crops, dependent upon environmental conditions and frost hardening of the crop (Reade *et al.*, 1999; Milner, Reade and Cobb, 2001). These timings coincide with the lowest values of GST activity recorded in this study. However as uptake and performance of all herbicides can be reduced during periods of dormant growth over the winter period, it is speculated that autumn applications of herbicides would be the most effective. Many commercially available herbicides are proposed for black-grass control up to GS39 (flag leaf ligule visible). The findings of this study indicate that the role of GSTs in terms of herbicide selectivity could be dependent on the developmental stage of the weed. It may be that herbicide recommendations need to be revised in order to avoid a decrease in efficacy and thus potentially greater yield losses. It must be acknowledged that these findings and speculations would not work in isolation and could only be used alongside other currently used methods of predicting spray windows such as growth stage of the crop and weed, season and environmental conditions and when considering reduced herbicide efficacy.

Further work is now required in this important area to isolate and identify GST isozymes as constitutive and/or inducible and to investigate their spectrum of activity. Investigation is also required with respect to GSH and S content and their effect on GST activity within the field. The precise effects of climatic factors and black-grass growth and development on GST activity also require further attention. This would enable further knowledge to be

acquired regarding the involvement of GSTs in endogenous metabolism, herbicide selectivity and resistance. The effect of S deficiency on black-grass is also worthy of further study and could be achieved through hydroponics determining whether S affects GSH pool size and thus GST activity in black-grass with respect to herbicide resistance.

3.5. CONCLUSION

A 2 year study was performed to investigate GST activity in untreated black-grass in the field with respect to herbicide resistance. Findings indicate there is a natural elevation of endogenous GST activity with changes in the growth of black-grass. It is proposed that increasing endogenous GST activity is required as part of an antioxidant defence system until tillering (GS30) has ceased. This is in order to protect developing plants from toxic endogenous substrates associated with environmental stress. This suggests that GSTs have direct cytoprotective activity during environmental changes and stress in conjunction with supporting normal plant development.

Current observation points towards innate GST activity within black-grass plants being partly responsible for reduced herbicide efficacy in the field. It is suggested that changes in GST activity normally occur during plant growth with changing climatic conditions. These observations lend further weight to the suggestion that the development of resistance in black-grass is in part due to evolution and elevation of GST activity. This points to the requirement that herbicide application must be early in the growing season to ensure maximal herbicide efficacy.

CHAPTER FOUR

AN INVESTIGATION INTO THE EFFECT OF TEMPERATURE ON PLANT GROWTH OF UNTREATED RESISTANT AND SUSCEPTIBLE BLACK-GRASS POPULATIONS

4.1. INTRODUCTION

It is well documented that abiotic environmental stresses such as temperature, water deficit and salinity restrict plant distribution and productivity. The number of stresses which plants are exposed to have increased with the activities of humans in the form of pollutants e.g. heavy metals, ozone, UV light and salinity in irrigation areas (Smallwood, Calvert and Bowles, 1999). Plants are sessile and have therefore developed various mechanisms, which induce immediate, acclimatory and adaptive responses allowing survival in different forms of extreme environmental stress as indicated by the novel observations of the previous chapter. These mechanisms exist in the form of an armoury of enzymatic and non-enzymatic antioxidant defences. Equilibrium exists between active oxygen species (AOS) production and degradation, which environmental stresses can disrupt at any time during the life cycle of the plant (Foyer and Fletcher, 2001). The molecular basis of these survival mechanisms are of particular interest when associated with herbicide tolerance, in particular with respect to the production of AOS in response to environmental stress. AOS accumulation acts as a signal for the need to acclimatise to changing environmental factors and it is postulated that the role of oxidative stress and antioxidants in abiotic stress responses may also play a part in plants becoming resistant to herbicides.

The aim of this study was to investigate the effect of temperature on plant growth and foliar antioxidant status in resistant and susceptible populations of black-grass. Observations of plant growth, protein content, GST activity and GSH concentration in plants subjected to different temperature regimes were recorded and the implications of the findings with respect to herbicide efficacy and application timing are discussed.

4.2. MATERIALS AND METHODS

4.2.1. Plant Material

The 2 populations of black-grass Herbiseed and Peldon were used in this study. Seeds were pre-germinated in Petri-dishes on filter paper (Whatman No. 1. Qualitative Circles 90 mm Dia, Whatman International Ltd, Maidstone, UK) soaked in distilled water in order to obtain uniform plant populations once transferred into the growth medium. To maximise germination, dishes were placed in a growth cabinet (S10H Conviron, Environmental Growth Cabinet, Winnipeg, Canada) under controlled conditions of 17°C, 14h day and 11°C, 10h night (± 5°C) as recommended by Moss (1996), with a photosynthetic photon flux density no lower than 120μmol m^{-2} sec^{-1}. Lighting was provided by fluorescent tubes (Sylvania VHO) and 9 60W tungsten bulbs. Germination occurred after 6-7 days, i.e. root length >0.5cm and coleoptile just emerging. The plants were transplanted into a soil-based growth medium (John Innes compost No.2) in 5 inch pots, watered well from below using laboratory tap water containing ions and returned to the growth cabinet under the same growth conditions. Plant populations were restricted to 6 plants per pot and 7 pots in total for each population. A constant temperature was set according to the particular regime being carried out utilising climatic data obtained for the previous chapter as guidelines. The average photosynthetic photon flux density at plant height throughout the series of experiments was 158μmol m^{-2} sec^{-1}. Pots were arranged in a fully randomised design and the plants were allowed to establish and mature to GS12/13 (2/3 true leaves) before subsequent harvests took place. Both susceptible and resistant plants were together and exposed to identical growth conditions. Apart from temperature, all other growth conditions were left unchanged for the duration of the experiments.

4.2.2. The effect of constant temperatures of 10°C and 25°C on plant growth of untreated resistant and susceptible black-grass.

4.2.2.1. Plant Material. Resistant and susceptible plants were transplanted into controlled conditions of a constant 10°C or 25°C and allowed to mature to GS12/13. Six plants of each population were then harvested by the removal of all above ground biomass at 7d intervals from GS12/13 for 7 weeks. Immediately post harvest and prior to storage, individual plant growth stage and plant height was documented for every harvested plant. The aerial foliage of each plant was also accurately weighed before being individually bagged and frozen in liquid nitrogen and stored at -80°C until required. Supplementary recordings of temperature, relative humidity and light readings were taken at every harvest.

4.2.3. The effect of temperature transfers on plant growth of untreated resistant and susceptible black-grass.

4.2.3.1. Plant Material – Temperature transfers 10°C – 17.5°C / 17.5°C - 10°C/ 10°C - 25°C / 25°C - 10°C. Resistant and susceptible plants were transferred into controlled conditions of a constant 10°C, 17.5°C or 25°C and allowed to mature to GS12/13. Six plants of each population were harvested by the removal of all above ground biomass at 7d intervals for 21 days from GS12/13 onwards. After the third harvest, temperature was immediately increased from 10°C to 17.5°C or 25°C or decreased from 25°C or 17.5°C to 10°C to simulate a temperature transfer. Plants were harvested every 4d for a period of 16d following the temperature change.

4.2.4. Determination of protein content in untreated resistant and susceptible black-grass exposed to different temperature regimes.

4.2.4.1. Plant Material and protein analysis. From every harvest, 3 of the 6 plants of each population were used for protein determination as described in Chapter 3.

4.2.5. Determination of glutathione *S*-transferase activity in untreated resistant and susceptible black-grass populations exposed to different temperature regimes.

The desalted plant extracts used for protein determination were subsequently prepared for GST assays against the model substrate CDNB, using the microplate adaptation of the previously reported assay of Reade and Cobb, (1999) as described in Chapter 3.

4.2.6. Determination of glutathione content in untreated resistant and susceptible black-grass populations exposed to different temperature regimes.

4.2.6.1. Plant Material and preparation of cell-free extracts. The remaining 3 plants of each population from every harvest were subsequently used for GSH determination. Cell-free extracts for further analysis were prepared using an adaptation of the method described by Griffith (1980). All chemicals were obtained from Sigma (Sigma-Aldrich Company Ltd, Dorset, England) unless otherwise stated. Approximately 1g sub-sample accurately weighed of whole plant tissue of each plant harvested was ground with a chilled pestle and mortar in 10ml 5% (w/v) aqueous 5-sulfosalicylic acid. The contents of the tube were then homogenised for 15 sec using a Silverson SL2 laboratory homogeniser (Silverson Machines, Chesham, Buckinghamshire, UK) at full speed. The resulting homogenate was centrifuged (Beckman Avanti 30) at 10,000g for 10 min at 4°C. Of the resulting supernatant 1.5ml was transferred into an Eppendorf tube and centrifuged

(Eppendorf Centrifuge 5415D, Eppendorf, Netheler, Heinz GmbH, 22331 Hamburg) at 13rcf for 5 min at room temperature. A 1ml aliquot of supernatant was neutralised by 1.5ml of 0.5M potassium phosphate buffer (pH 7.5) containing 1mM sodium ethylenediaminetetraacetic acid (Na_2EDTA). Samples were assayed as soon as possible post extraction due to the potential loss of or change in oxidation state in intracellular GSH (Baker, Cerniglia and Zaman, 1990).

4.2.6.2. Glutathione determination. Cell-free plant extracts were assayed using an adaptation of the microplate method as described by Baker *et al.*, (1990). The reaction mixture was prepared by mixing together 1mM 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) dissolved in 5ml 0.5M potassium phosphate buffer (pH 7.5) containing 1mM Na_2EDTA, 1mM NADPH dissolved in 5ml distilled water, 5.75ml potassium phosphate buffer (pH 7.5) containing 1mM Na_2EDTA and 0.1ml glutathione reductase (200 units ml^{-1} distilled water) which was stored on ice. The amount of reaction mixture required varied according to the number of samples being processed. To construct a standard curve, a GSH stock solution was prepared by dissolving 1mM GSH in 10ml of distilled water. This was diluted to the required concentration of 8nmol ml^{-1} by taking 80µl of stock and mixing it in 9.9ml distilled water. A standard curve was determined using differing concentrations of GSH standards at a range of concentrations (100 – 400 pmol) (50µl per well, 4 wells per standard) increased to a final assay volume of 150µl by the addition of 100µl of reaction mixture which initiated the reaction. The rate at which DTNB was oxidised to 2-nitro-5-thiobenzoic acid was followed at 405nm in a Bio-Rad Benchmark microplate reader utilising Microplate Manager v5.0 software. Readings were taken every 2 min for 10 min, during which the velocity of reaction remained linear and a standard curve was obtained. This assay was carried out using 50µl cell-free extract to which 100µl reaction mixture was added. The GSH concentration of individual plants was calculated using the equation

obtained from the standard curve. Replacing extract with an equal volume of sulfosalicylic acid assessed blank samples.

4.2.7. Data analysis

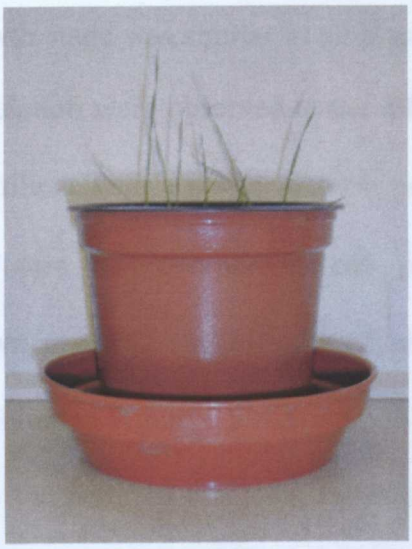
All morphological growth data (height and foliage fresh weight) was expressed as centimetres (cm) and grams (g) and based on observations of 6 plants per population at each harvest point. Statistical analyses of these values were carried out using Genstat 5 Release 4.1. T-tests were carried out to test the null hypothesis of zero mean difference at the 95% confidence level ($P<0.05$ = significant, $P>0.05$ = non-significant) that the height and fresh weight of individual plants was not affected by different temperatures. Values for protein content, GST activity and GSH content, comparing and contrasting between different temperatures and between populations were based on observations of 3 plants per population at each harvest point. These values were expressed as protein content g^{-1} fwt (mg g^{-1} fwt), GST specific activity (µmol CDNB min^{-1} mg^{-1} total protein), GST activity (µmol CDNB min^{-1} g^{-1} fwt) and GSH concentration (nmol g^{-1} fwt). Statistical analyses of these values by factorial analysis of variance were calculated and analysed using Genstat and in addition t-tests were used to test the null hypothesis of zero mean difference at the 95% confidence level comparing the response of different populations at different growth stages. In all cases, standard errors were calculated.

4.3.1. The effect of temperature on plant growth of untreated resistant and susceptible black-grass.

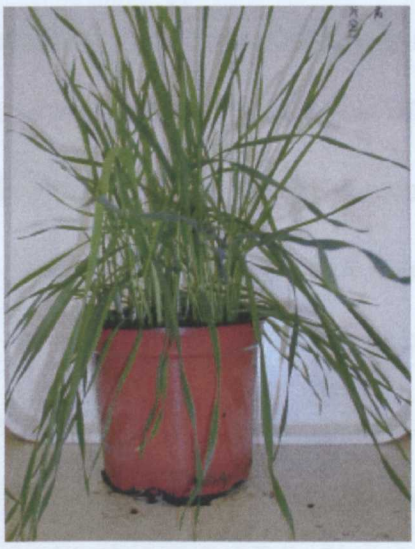
As expected, temperature was observed to have a developmental effect on the growth of resistant and susceptible plants in each study, as illustrated in Figure 4.1. with all plants developing more rapidly at higher temperatures. Morphological responses to temperature were also observed in the plants. In every study, plant height and fresh weight of the populations significantly increased ($P<0.001$) over time. The average temperature and relative humidity of each study is presented in Table 4.1. and shows that a constant environment was maintained.

Table 4.1. Average growth temperature (°C) and relative humidity (%) of each temperature study conducted. Values are means ±SE values, where $n = 7$ observations.

Study	Average temperature (°C)	Average relative humidity (%)
10°C constant	10.0 (±0.0)	75.4 (±0.0)
25°C constant	25.0 (±0.0)	99.0 (±0.0)
10°C - 17.5°C transfer	10.1 (±0.2) 17.5 (±0.0)	86.3 (±2.4) 75.0 (±3.5)
17.5°C - 10°C transfer	17.6 (±0.0) 10.0 (±0.0)	98.3 (±0.7) 95.3 (±0.9)
10°C - 25°C transfer	10.0 (±0.1) 25.0 (±0.0)	77.7 (±0.3) 99.0 (±0.0)
25°C - 10°C transfer	24.9 (±0.0) 10.0 (±0.0)	99.0 (±0.0) 72.3 (±4.0)



Week 1 10°C



Week 7 10°C

Week 1 25°C

Week 7 25°C

Figure 4.1. The effect of constant growing temperatures of 10°C and 25°C on the growth of untreated resistant and susceptible black-grass populations. Photographs of representative plants are presented and are typical of both the resistant and susceptible populations. ⟷ = 10 cm

4.3.1.1. The effect of constant growth temperatures of 10°C and 25°C on plant growth of resistant and susceptible black-grass. As expected plants of each population grown at 25°C matured more rapidly than those grown at 10°C. Between GS12 and 22, growth stage was similar in all plants at both temperatures. However, differences in each population were observed at the different temperatures with respect to numbers of leaves and tillers, with a greater number present in plants grown at 25°C as clearly demonstrated in Figure 4.1. Morphologically, plants grown at 10°C appeared more erect than those grown at 25°C suggesting that high temperature induces developmental responses as exhibited in Figure 4.1. This is confirmed in the observation of senescence in leaves 1 and 2 in the form of chlorosis as early as GS22 in all plants grown at 25°C. In contrast, senescence in these leaves in plants grown at 10°C was not observed until GS27, at the final harvest point.

No significant difference ($P>0.05$) between plant height and resistant and susceptible black-grass was observed at 10°C. However at 25°C, Herbiseed was significantly taller ($P<0.05$) than Peldon between GS22 and 27. The response within the individual populations revealed a significant difference ($P=/<0.05$) in plant height between GS12 and GS21/22, which was transient. Random differences ($P<0.05$) at later growth stages were observed, but by the final harvest, there was no significant difference ($P<0.05$) within the populations. No significant difference ($P>0.05$) between plant fresh weight and the resistant and susceptible populations were observed at either temperature. Within individual populations, plants grown at 10°C were heavier.

4.3.1.2. The effect of temperature transfers on plant growth of resistant and susceptible black-grass. Temperature transfers induced developmental changes in plants

of both populations. Plants transferred from 10°C to 17.5°C immediately developed more rapidly with more leaves and tillers being produced with these changes in plants transferred from 10°C to 25°C being even more pronounced. However, plants did not adapt and respond immediately, but rapid development was observed from 8d post transfer (GS26) with plants observed to be near the end of tillering (GS30) on completion of the study. In contrast, plants transferred from 25°C or 17.5°C to 10°C, continued to develop, but on completion of the studies they were observed to be less advanced at GS26. The effects of the transfer studies on plant growth are illustrated in Figures 4.2. - 4.5. demonstrating clear morphological responses. Plants grown initially at 10°C possessed more upright stance than those at 17.5°C or 25°C as can be seen by comparing and contrasting pre-temperature transfer photographs in Figures 4.2. and 4.4. to those in Figures 4.3. and 4.5. At higher temperatures plants possessed few erect leaves from GS22 onwards with those grown at 25°C also exhibiting senescence in leaves 1 and 2. Plants transferred from 10°C to higher temperatures exhibited morphological responses 4d post transfer with leaves losing upright stance and senescence clearly beginning on leaves 1 and 2. Some plants transferred to 25°C also exhibited wilting as can be seen in Figure 4.4., despite a regular, non-limiting water supply. In contrast, plants transferred from 25°C to 10°C did not visibly alter in appearance immediately post transfer but did recover some upright stance 12d post transfer, although leaves 1 and 2 were completely senescent by this point as demonstrated in Figure 4.5. Plants transferred from 17.5°C exhibited senescence in leaves 1 and 2 from GS23, 4d post transfer to 10°C and by 16d post transfer some upright stance had been recovered as shown in Figure 4.3.

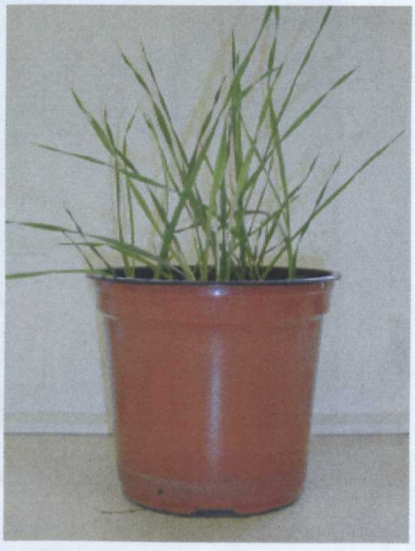
No significant differences ($P>0.05$) between the plant height of the resistant and susceptible populations were observed between GS12 and GS23/24, with the exception of

the height of Herbiseed plants being significantly higher ($P<0.05$) than Peldon at GS22 in the 17.5°C - 10°C study. However, temperature transfers induced clear developmental responses. Plants initially grown at 10°C and transferred to the higher temperatures exhibited significant differences ($P<0.01$) in plant height 4d post temperature transfer which was most pronounced in the 10°C - 25°C study. In the 10°C – 17.5°C, study these differences were transient. The larger transfer of 10°C - 25°C induced significant ($P<0.01$) plant height differences between Herbiseed and Peldon which were maintained from GS25 to GS26 covering 4d to 16d post temperature transfer. In contrast, no plant height response was exhibited in the populations transferred from 17.5°C - 10°C. Few differences in the 25°C - 10°C study were exhibited with the exception that the height of Herbiseed was significantly higher ($P<0.05$) than Peldon at GS22 and between GS26 and GS28. The response within the individual populations revealed small variations. Significant differences ($P<0.05$) in plant height within the populations at GS11-12 were observed, with those grown at 17.5°C and 25°C being taller. The responses within the populations to each study were similar with the populations increasing in height pre-temperature transfer and in the majority of cases a decrease in height was observed 4d post transfer which was transient.

No significant differences ($P>0.05$) between the mean plant fresh weight of the resistant and susceptible populations were observed, with 2 exceptions. These were between GS25 and GS26 (10°C - 25°C), where fresh weight of Herbiseed was significantly greater ($P<0.05$) than Peldon. No fresh weight responses to the transfers were observed. However, the mean fresh weight of all the populations was observed to be significantly lower ($P<0.05$) when initially grown at 25°C or 17.5°C and transferred to 10°C than in the reverse studies. The response within the individual populations to the temperature transfers revealed that mean fresh weight was significantly greater ($P<0.05$) in every

population grown at 10°C and transferred to 17.5°C or 25°C, 4d post temperature transfer (GS23-26) as opposed to those grown in the reverse studies. These differences were maintained in every population.

10°C GS12/13

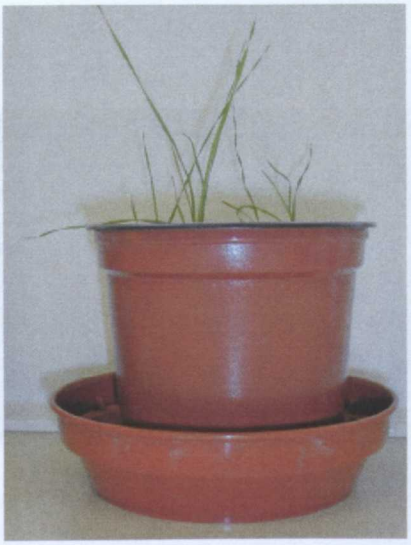


10°C immediately pre-transfer to 17.5°C

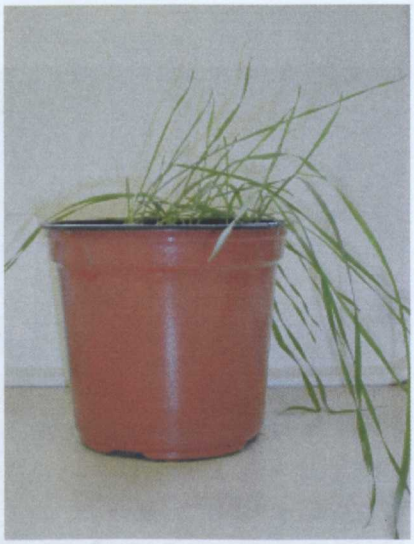
4d post transfer to 17.5°C

16d post transfer to 17.5°C

Figure 4.2. The effect of a temperature transfer from 10°C to 17.5°C on the growth of untreated resistant and susceptible black-grass populations. Photographs of representative plants are presented and are typical of both the resistant and susceptible populations. ⟷ = 10cm



17.5°C GS12/13



17.5°C immediately pre-transfer to 10°C

4d post transfer to 10°C

16d post transfer to 10°C

Figure 4.3. The effect of temperature transfer from 17.5°C to 10°C on the growth of untreated resistant and suscpetible black-grass populations. Photographs of representative plants are presented and are typical of both the resistant and susceptible populations. ⟷ = 10 cm.

10°C GS12/13

10°C immediately pre-transfer to 25°C

4d post transfer to 25°C

16d post transfer to 25°C

Figure 4.4. The effect of temperature transfer from 10°C to 25°C on the growth of untreated resistant and susceptible black-grass populations. Photographs of representative plants are presented and are typical of both the resistant and susceptible populations. ◄——► = 10 cm

25°C GS12/13

25°C immediately pre-transfer to 10°C

4d post transfer to 10°C

16d post transfer to 10°C

Figure 4.5. The effect of temperature transfer from 25°C to 10°C on growth of untreated resistant and susceptible black-grass populations. Photographs of representative plants are presented and are typical of both the resistant and susceptible populations. ⟷ = 10cm.

Tables 4.2. to 4.5. detail the results of statistical analyses carried out using analysis of variance to interpret the results for protein content (mg g^{-1} fwt), GST specific activity (μmol CDNB min^{-1} mg^{-1} total protein), GST activity (μmol CDNB min^{-1} g^{-1} fwt) and GSH concentration (nmol g^{-1} fwt). The results relating to these analyses are then discussed in further detail. Please see the reverse of this page for a key relating to the tables.

Key:

Regime

1 - 10°C and 25°C constant

2 – 10°C and 17.5°C

17.5°C and 10°C

3 - 10°C – 25°C

25°C – 10°C

Popn – population **GS** – growth stage **Temp** – temperature

CV – coefficient of variation

X – non-significant ($P>0.05$) **√** - significant ($P<0.05$)

Table 4.2. Analysis of variance relating to the effect of temperature on protein content (mg g^{-1} fwt) of untreated resistant and susceptible populations of black-grass .

Regime	Population	Temperature	Growth Stage	Pop^n.Temp	Pop^n.GS	Temp.GS	Pop^n.Temp.GS	CV (%)
1	X	√ P<0.001	√ P<0.001	X	X	√ P<0.001	X	27.2
2	√ P<0.001	√ P<0.001	√ P<0.001	X	√ P<0.001	√ P<0.001	X	14.2
3	X	√ P<0.001	√ P<0.001	X	X	√ P<0.001	X	29.2

Table 4.3. Analysis of variance relating to the effect of temperature on GST specific activity (µmol CDNB min^{-1} mg^{-1} total protein) of untreated resistant and susceptible populations of black-grass.

Regime	Population	Temperature	Growth Stage	Pop^n.Temp	Pop^n.GS	Temp.GS	Pop^n.Temp.GS	CV (%)
1	√ P<0.001	√ P<0.001	√ P<0.001	√ P<0.001	X	√ P<0.01	√ P<0.05	26.8
2	√ P<0.001	√ P<0.001	√ P<0.001	√ P<0.01	X	√ P<0.001	√ P<0.05	32.1
3	√ P<0.001	√ P<0.001	√ P<0.001	√ P<0.05	√ P<0.01	√ P<0.001	√ P<0.05	9.7

Table 4.4. Analysis of variance results relating to the effect of temperature on GST activity (μmol CDNB min^{-1} g^{-1} fwt) of untreated resistant and susceptible populations of black-grass.

Regime	Population	Temperature	Growth Stage	Pop^{R}.Temp	Pop^{R}.GS	Temp.GS	Pop^{R}.Temp.GS	CV (%)
1	√ P<0.001	√ P<0.001	√ P<0.001	√ P<0.001	√ P<0.001	√ P<0.001	√ P<0.01	21.7
2	√ P<0.001	X	√ P<0.001	√ P<0.01	√ P<0.05	√ P<0.001	√ P<0.01	10.4
3	√ P<0.05	√ P<0.001	√ P<0.001	X	√ P<0.05	√ P<0.001	X	10.6

Table 4.5. Analysis of variance results relating to the effect of temperature on glutathione content (nmol g^{-1} fwt) of untreated resistant and susceptible populations of black-grass.

Regime	Population	Temperature	Growth Stage	Pop^{R}.Temp	Pop^{R}.GS	Temp.GS	Pop^{R}.Temp.GS	CV (%)
1	X	√ P<0.001	√ P<0.001	X	√ P<0.01	√ P<0.01	X	22.2
2	X	√ P<0.01	√ P<0.01	X	X	√ P<0.01	X	25.0
3	X	√ P<0.01	√ P<0.01	X	√ P<0.05	√ P<0.01	X	4.6

4.3.2. Protein content and GST specific activity in resistant and susceptible black-grass grown at constant temperatures and exposed to temperature transfers.

4.3.2.1. Protein content. Statistical analysis (Table 4.2.) indicated highly significant differences ($P<0.001$) between growth temperatures (constant and transfers) and growth stage at harvest. There was however, no difference ($P>0.05$) between the two populations under the different regimes with the exception of the 10°C – 17.5°C / 17.5°C - 10°C studies. No interactions ($P>0.05$) were observed between the populations and temperature or population and growth stage (exception 10°C – 17.5°C / 17.5°C - 10°C studies with respect to the latter). Additionally, there was no 3-way interaction ($P>0.05$) between population, temperature and growth stage at harvest. In contrast, a highly significant interaction ($P<0.001$) was observed between temperature and growth stage at harvest under all the regimes. The results of these studies indicated that protein synthesis is tied into the growth needs of both resistant and susceptible plants and their stage of development, for example, plants transferred from high to low temperatures as illustrated in Figure 4.6.

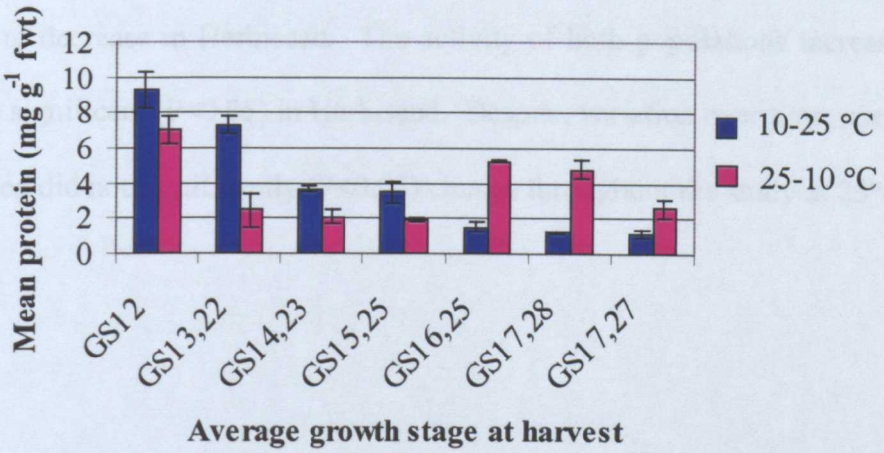


Figure 4.6. Mean protein content (mg g^{-1} fwt) of the resistant black-grass population Peldon exposed to large temperature transfers. Protein values are means ±SE values, where $n = 3$ plants. Error bars are included where they exceed the symbol size.

4.3.2.2. Glutathione *S*-transferase specific activity in resistant and susceptible black grass grown at 10°C and 25°C. Analysis of variance (Table 4.3.) indicated a highly significant difference ($P<0.001$) between populations, temperature and growth stage at harvest. Significant interactions ($P<0.05$) were also observed between population and temperature, temperature and growth stage and population, temperature and growth stage. In contrast, there was no interaction ($P>0.05$) between population and growth stage at harvest.

As expected Peldon, exhibited higher GST specific activity than Herbiseed at both temperatures. At 10°C specific activity of the populations was observed to decrease over time, but no significant ($P<0.05$) changes were observed, with 2 exceptions. Specific activity was observed to be higher at 25°C and activity of Peldon was significantly higher ($P<0.05$) than Herbiseed for the duration of the study as illustrated in Figure 4.7. In both populations specific activity increased from GS21 and peaked at GS23 which was significant ($P<0.05$) in Peldon and subsequently decreased, which was again significant ($P<0.05$) in Peldon. Between GS25 and GS26 activity of Peldon remained constant but continued to decrease in Herbiseed. The activity of both populations increased at GS27, which was significant ($P<0.05$) in Herbiseed. Despite, variation over time, specific activity of Herbiseed did not significantly ($P<0.05$) change throughout the study at 25°C in contrast to Peldon.

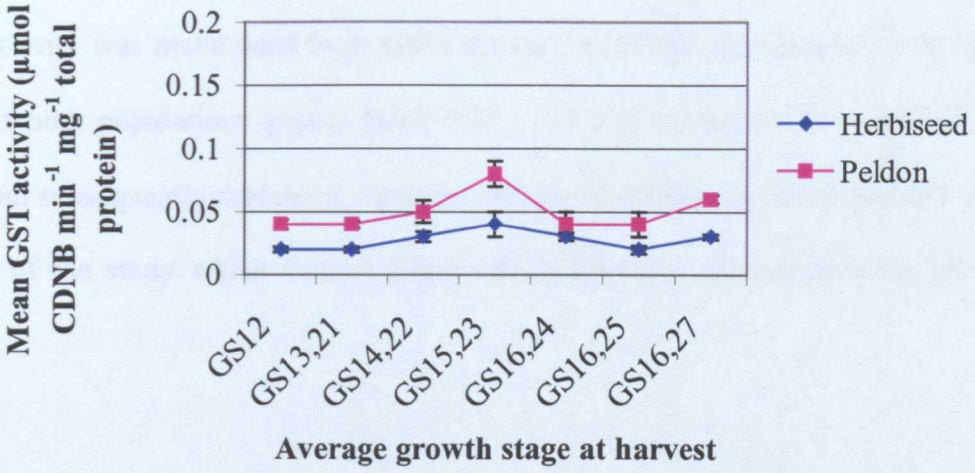


Figure 4.7. Mean GST specific activity (µmol CDNB min^{-1} mg^{-1} total protein) in untreated resistant and susceptible black-grass grown at 25°C. GST specific activity values are means ±SE values, where n = 3 plants. Error bars are included where they exceed the symbol size.

4.3.2.3. Glutathione *S*-transferase specific activity in resistant and susceptible black-grass populations exposed to temperature transfers. Statistical analysis (Table 4.3.) indicated a highly significant difference ($P<0.001$) between the populations, temperature regimes and growth stage at harvest. This was accompanied by significant interactions ($P<0.05$) between population and temperature, temperature and growth stage and population, temperature and growth stage. A significant interaction ($P<0.01$) between population and growth stage was observed under the 10°C – 25°C / 25°C - 10°C regime but this was non-significant ($P>0.05$) under the 10°C – 17.5°C / 17.5°C - 10°C regime.

No responses to temperature transfer from 10°C – 17.5°C were observed but in contrast clear responses to the 10°C – 25°C transfer were exhibited as demonstrated in Figure 4.8. Activity of Peldon was consistently higher than that of Herbiseed in both studies. Under the 10°C – 17.5°C regime, activity of Peldon was significantly ($P<0.05$) higher than

Herbiseed between GS26 and GS28. However, under the 10°C – 25°C regime, this elevated activity was maintained from GS12 through to GS26 (completion of the study). Activity of both populations grown from 10°C – 17.5°C increased between GS12 and GS21/22 but subsequently decreased. Specific activity of Peldon remained constant for the remainder of the study whilst that of Herbiseed continually, non-significantly ($P>0.05$) decreased.

Both populations responded when grown at 10°C and transferred to 25°C. Activity remained constant in both populations between GS12 and GS24 with no differences ($P>0.05$) between populations observed. Specific activity of Peldon remained constant between 4d and 8d post temperature transfer as that of Herbiseed decreased (Figure 4.8.) As a result, activity of Peldon was significantly higher ($P<0.05$) than Herbiseed between GS24 and GS25 despite no immediate response in any of the populations to the temperature change, leading to a resistant to susceptible ratio of 2 at GS24. Activity of both populations increased 8d post transfer (GS25) as demonstrated in Figure 4.8. Despite this increase being non-significant ($P>0.05$), it led to larger significant ($P<0.05$) differences between the populations with the resistant to susceptible ratio at a maximum of 2.67 at this point. Activity continued to increase and highlight these differences but subsequently decreased in both populations' 16d post transfer, resulting in a complete collapse in the resistant to susceptible ratio to 1.25. This collapse was gradual over the final 3 harvest points ($2.67 \rightarrow 1.8 \rightarrow 1.25$) and resulted in no significant difference ($P>0.05$) in activity between the populations on completion of the study.

The effect of temperature transfer from high to low induced clear responses in GST specific activity under both regimes as illustrated in Figures 4.8. with activity of Peldon consistently higher than that of Herbiseed. Transfer from 17.5°C to 10°C induced a pronounced effect in Peldon as illustrated in Figure 4.8. No significant difference in

activity between the populations was observed at GS12 but it increased between GS12 and GS22, after which activity of Peldon continued to increase but that of Herbiseed significantly and continuously decreased ($P<0.05$) over time (Figure 4.8). A significant difference ($P<0.05$) between Herbiseed and Peldon was present at GS21 with a resistant to susceptible ratio of 1.4 increasing to 2.67 by GS22. However, 4d post temperature transfer (GS24) activity of Peldon significantly decreased ($P<0.01$) and the ratio collapsed to 1.0 (Figure 4.8). Activity of Peldon subsequently remained constant between GS24 and GS25 whilst that of Herbiseed decreased, initiating a resistant to susceptible ratio of 1.5. Specific activity of Herbiseed remained constant through to the study completion as that of Peldon decreased to bring the ratio back to 1. However, 16d post transfer, activity of Peldon significantly increased ($P<0.05$) resulting in a significant difference ($P<0.05$) between the populations and a resistant to susceptible ratio of 2. No significant difference ($P>0.05$) in the specific activity of Herbiseed or Peldon at the start of the study compared to completion was observed.

The effect of temperature transfer from 25°C to 10°C induced a very pronounced response in Peldon but to a much lesser extent in Herbiseed as can be seen and compared in Figure 4.8. with activity of Peldon again higher than Herbiseed, but which was transient. Activity of Herbiseed significantly ($P<0.01$) decreased between GS12 and GS21 and then increased between GS21 and GS22, whereas that of Peldon remained constant. Specific activity of both populations significantly increased ($P<0.001$) 4d post transfer as illustrated in Figure 4.8. The resistant to susceptible ratio between Herbiseed and Peldon prior to transfer was present but reached a maximum of 2.8 at GS23 (4d post transfer). However, between 4d and 8d post transfer, activity of both populations significantly decreased ($P<0.05$) (Figure 4.8). leading to a collapse in the ratio which continued for the remainder of the study as specific activity remained constant.

Specific activity within the individual populations was higher in plants grown initially at 25°C or 17.5°C and transferred to 10°C. The response within Herbiseed to the different temperature regimes was insignificant, although as expected significant differences ($P<0.05$) between activity of plants under different temperatures were observed, there were no marked effects. Activity within Peldon was similar under the different regimes with activity in plants initially grown at 17.5°C higher than those at 10°C, but the transfer led to significant decreases in activity ($P<0.05$) resulting in no further differences ($P>0.05$). Specific activity of Peldon was observed to be higher in plants grown under the 25°C to 10°C regime for the first 4 harvest points. However, a role reversal was observed post transfer, as activity was higher in plants transferred from 10°C to 25°C. The activity of these plants increased as that of those transferred to 10°C decreased as illustrated in Figure 4.9.

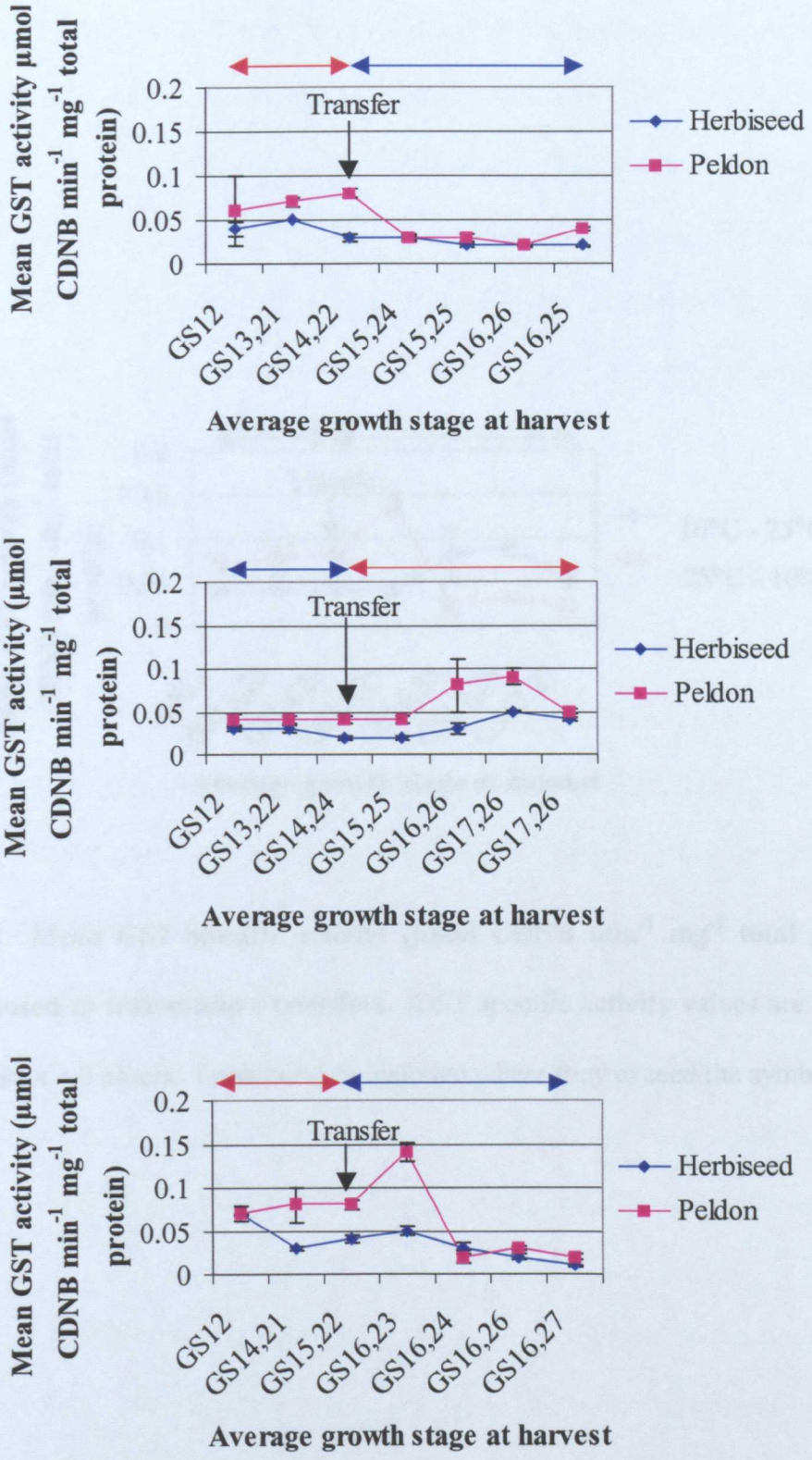


Figure 4.8. Mean GST specific activity (µmol CDNB min^{-1} mg^{-1} total protein) in untreated resistant and susceptible black-grass populations exposed to temperature transfers. GST specific activity values are means ±SE values, where $n = 3$ plants. Error bars are included where they exceed the symbol size.

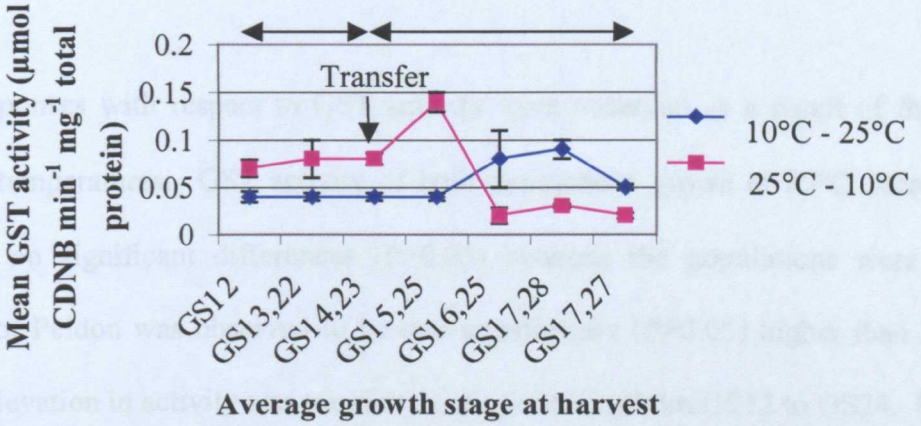


Figure 4.9. Mean GST specific activity (µmol CDNB min^{-1} mg^{-1} total protein) in Peldon exposed to temperature transfers. GST specific activity values are means ±SE values, where $n = 3$ plants. Error bars are included where they exceed the symbol size.

4.3.3. Glutathione *S*-transferase activity in untreated resistant and susceptible black-grass populations grown at constant temperatures of 10°C and 25°C.

Analysis of variance (Table 4.4.) revealed highly significant differences ($P<0.001$) between populations, temperature and growth stage at harvest. All interactions were significant ($P<0.01$) – population and temperature, population and growth stage, temperature and growth stage and population, temperature and growth stage.

Clear responses with respect to GST activity were observed as a result of the different growing temperatures. GST activity of both populations grown at 10°C decreased over time but no significant differences ($P>0.05$) between the populations were observed. Activity of Peldon was observed to be non-significantly ($P>0.05$) higher than Herbiseed, but this elevation in activity was transient, only persisting from GS12 to GS24. In contrast, activity of Peldon grown at 25°C was significantly higher ($P<0.05$) than Herbiseed and was maintained from GS12 to GS28 with a resistant to susceptible ratio ranging between 1.6 and a maximum of 2.71 at GS21. As at 10°C activity of both populations decreased over time up to GS24 when subsequent activity increased which was significant ($P<0.01$) in Herbiseed at GS28. A significant difference ($P<0.05$) in GST activity of the populations at the beginning of the studies compared to activity on completion was observed.

GST activity and change over time within the individual populations was similar at both temperatures but exhibited some variation between the 2 temperatures, which was occasionally significant ($P<0.05$) with GST activity being higher at 25°C in Peldon.

4.3.4. Glutathione *S*-transferase activity in resistant and susceptible black-grass populations exposed to temperature transfers.

Statistical analysis (Table 4.4.) revealed significant differences ($P<0.05$) between populations, temperature regimes and growth stage at harvest with the exception of the

10°C -17.5°C / 17.5 - 10°C regime and temperature which was non-significant (P>0.05). All interactions were significant (P<0.05) for the 10°C -17.5°C / 17.5 - 10°C regime but only the interactions between population and growth stage and temperature and growth stage were significant (P<0.05) under the 10°C - 25°C / 25 - 10°C regime.

4.3.4.1. Temperature transfers from 10°C to 17.5°C or 25°C. No responses to temperature transfer were exhibited and a decreasing trend in GST activity was observed. As expected, there were clear differences between Peldon and Herbiseed with activity of Peldon persistently and sometimes significantly (P<0.05) higher than Herbiseed from GS12 through to the penultimate or final harvest point.

4.3.4.2. Temperature transfers from 25°C or 17.5°C to 10°C. In contrast to the above studies, clear responses to transfers from a high to low temperature were observed particularly in Peldon, as can be seen in Figure 4.10. Little response to the 17.5°C to 10°C transfer was exhibited by Herbiseed but this was contrasted by the clear response of Peldon as seen in Figure 4.10. Activity of both populations was constant between GS12 and GS22 with the exception of activity of Herbiseed, which significantly decreased (P<0.05) between GS21 and GS22. GST activity of Peldon was significantly higher (P<0.05) than Herbiseed with an accompanying resistant to susceptible ratio ranging between 2.08 to a maximum of 3 at GS22. but these ratios and elevated activity were transient. Activity of Peldon significantly decreased (P<0.01) with an accompanying collapse of the resistant to susceptible ratio between GS22 and GS24 as a result of the temperature transfer (Figure 4.10). In contrast, activity of Herbiseed, significantly increased (P<0.05) resulting in no significant difference (P>0.05) in activity between the 2 populations which was maintained. The GST activity of both populations remained constant for the remainder of the study with the exception of activity of Herbiseed, which significantly decreased (P<0.05) between GS24 and GS25 (4d to 8d post transfer).

The 25°C to 10°C regime induced clear responses in both populations as illustrated in Figure 4.10. GST activity of Peldon was significantly ($P<0.05$) (exception GS23) and persistently higher than Herbiseed and maintained from GS12 through to GS28. A resistant to susceptible ratio of 1.82 was present at GS12, which increased to 2.83 prior to temperature transfer. This ratio decreased to 2, 4d-post transfer and subsequently collapsed to 1 at GS24 (8d-post transfer). However, a ratio of 1.86 was observed at GS26 (12d-post transfer) but this decreased to 1.33 on completion of the study. The responses of the populations were similar as activity significantly decreased ($P<0.01$) between GS12 and GS21. Subsequent variations in activity pre-temperature transfer were non-significant ($P>0.05$). As a result of the temperature transfer, immediate responses in the form of significant increases ($P<0.05$) in activity were observed in the populations 4d post transfer (GS23) as demonstrated in Figure 4.10. However, this elevation in activity was transient as it was observed to subsequently decrease between 4d and 8d post transfer, which was significant ($P<0.05$) in Peldon. This decrease also resulted in a complete collapse of the resistant to susceptible ratio to 1.0. Activity of Herbiseed continued to decrease for the remainder of the study, which was significant ($P<0.05$) between GS25 and GS27. In contrast, GST activity of Peldon non-significantly increased ($P>0.05$) between GS24 and GS26 (8d to 12d post transfer) prior to significantly decreasing ($P<0.001$) between GS26 and GS27. Activity of both populations was significantly lower ($P<0.01$) on completion of the study compared to the beginning.

The response within individual populations to the 10°C – 17.5°C / 17.5°C – 10°C induced non-significant ($P>0.05$) trends in GST activity i.e. activity of Peldon in the 10°C – 17.5°C study was similar to that exhibited in the reverse study (17.5°C – 10°C). GST activity followed a decreasing trend in the populations initially grown at 10°C and transferred to 25°C with no metabolic response to the transfer observed. However, plants initially grown

at 25°C exhibited initial decreases in activity before increasing post temperature transfer to 10°C and subsequently decreasing again. This resulted in significant differences ($P<0.05$) in GST activity within the populations prior to temperature transfer and 4d to 8d post transfer, after which no significant difference ($P>0.05$) in activity was observed. Activity was observed to be higher in plants grown initially at 10°C, however, post temperature transfer, GST activity was persistently higher in plants transferred from 25°C to 10°C.

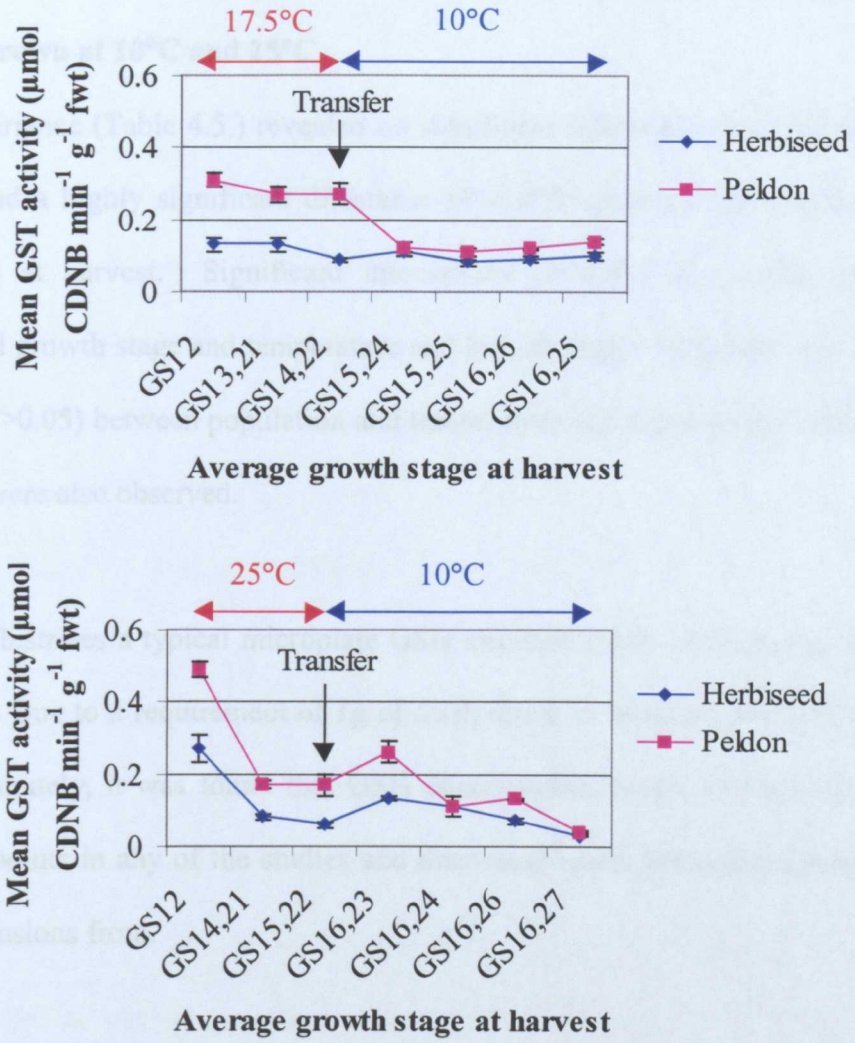


Figure 4.10. Mean GST activity (µmol CDNB min^{-1} g^{-1} fwt) in resistant and susceptible populations of black-grass exposed to temperature transfers. GST values are means ±SE values, where n = 3 plants. Error bars are included where they exceed the symbol size.

4.3.5. Glutathione content in untreated resistant and susceptible black-grass populations grown at 10°C and 25°C.

Analysis of variance (Table 4.5.) revealed no significant difference ($P>0.05$) between the populations and a highly significant difference ($P<0.001$) between the temperatures and growth stages at harvest. Significant interactions ($P<0.01$) were observed between population and growth stage and temperature and growth stage. However, non-significant interactions ($P>0.05$) between population and temperature and population, temperature and growth stage were also observed.

Figure 4.11. illustrates a typical microplate GSH standard curve and samples which have been assayed. Due to a requirement of 1g of fresh tissue to carry out the GSH extraction and assay accurately, it was found that GSH concentration could not be determined for early harvest points in any of the studies and thus insufficient data was acquired to draw accurate conclusions from.

GSH concentration of the populations non-significantly ($P>0.05$) decreased over time at both 10°C and 25°C, but no significant difference ($P>0.05$) between the populations was observed. Mean GSH concentration was significantly lower ($P<0.001$) in the populations grown at 25°C. A significant difference ($P<0.05$) in the GSH concentration of the populations grown at 10°C at the end of the study compared to the beginning was observed, but was not exhibited in plants grown at 25°C. Within the individual populations, a decrease in GSH concentration over time was observed at both temperatures, with GSH concentration being significantly higher ($P<0.05$) in the populations grown at 10°C.

4.3.6. Glutathione content of resistant and susceptible black-grass populations exposed to temperature transfers.

Statistical analysis (Table 4.5.) revealed no significant difference ($P>0.05$) between the populations but in contrast, significant differences ($P<0.01$) between the temperature regimes and growth stage at harvest. Similar to the constant temperature study, significant interactions ($P<0.05$) between population and growth stage (exception the 10°C - 17.5°C / 17.5°C - 10°C regime) and temperature and growth stage were observed. As previously, no significant interactions ($P>0.05$) between population and temperature and population, temperature and growth stage were observed for the regimes.

GSH concentration of the populations transferred from 10°C to 17.5°C or 25°C all non-significantly ($P>0.05$) decreased over time with no metabolic responses to the temperature transfers observed. No significant difference ($P>0.05$) between the populations was observed. However, no data points prior to temperature transfer from 25°C or 17.5° to 10°C were obtained for the populations. Changes in GSH concentration post-temperature transfer were observed under both regimes as demonstrated in Figure 4.12. No significant differences in GSH concentration between the populations were observed.

Clear variation in GSH concentration of the populations was exhibited under the 17.5°C to 10°C as illustrated in Figure 4.12. GSH concentration of both populations increased between GS22 and GS24 (4d post transfer) which was significant ($P<0.05$) in Herbiseed but subsequently non-significantly ($P>0.05$) decreased in both populations between GS24 and GS25 (4d to 8d post transfer). Prior to completion of the study GSH significantly increased ($P<0.05$) in both populations before decreasing again, which was significant ($P<0.01$) in Herbiseed. Between GS24 and GS26, GSH concentration of Herbiseed was observed to be significantly higher ($P<0.05$) than Peldon, but this was transient and not

GSH standard curve microplate

GSH samples microplate

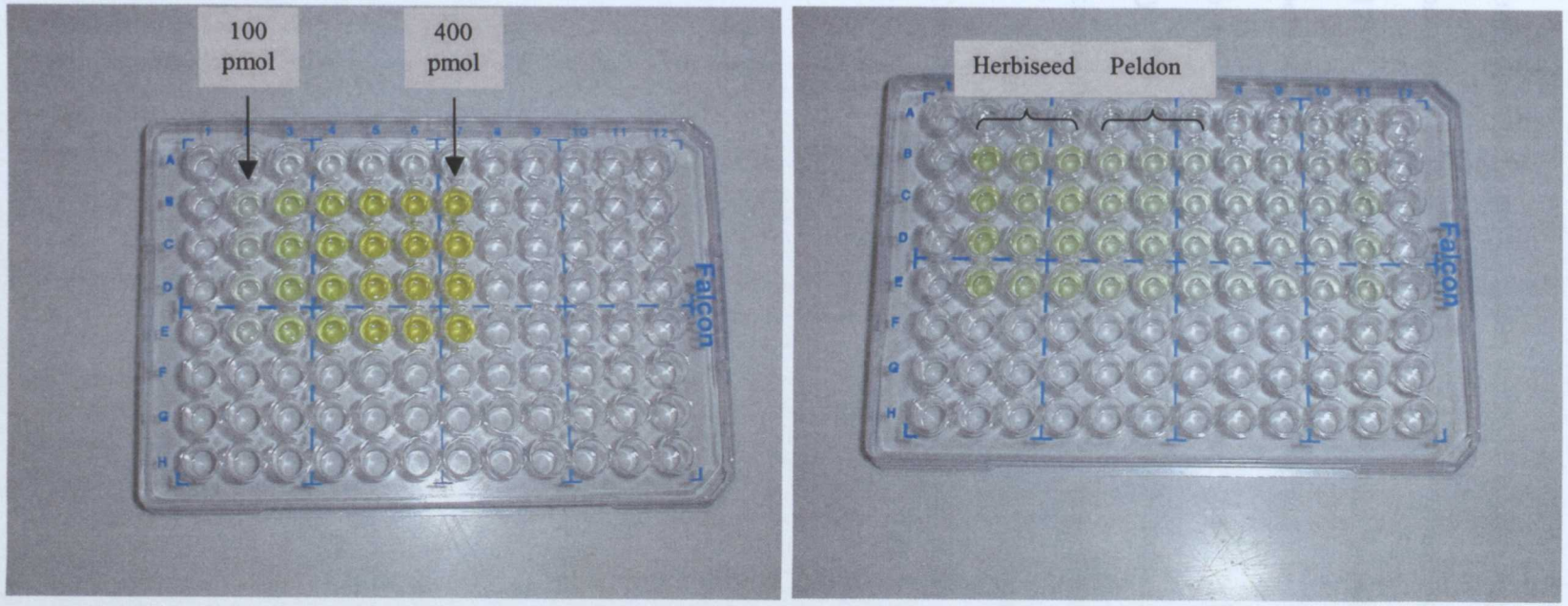


Figure 4.11. Typical glutathione standard curve and samples microplates created for the determination of glutathione in untreated resistant and susceptible black-grass. Glutathione concentrations for the standard curve range from 100 – 400pmol.

present on completion of the study. Overall, the GSH concentration of the 2 populations was higher than in the previous transfer study from 10°C to 17.5°C.

Plants of both populations grown under the 25°C to 10°C regime exhibited initial increases in GSH concentration post temperature transfer which was observed 4d post transfer (Figure 4.12). Mean GSH concentration of the populations significantly increased ($P<0.05$) between 4d and 8d-post temperature transfer (GS23 – GS24), which was followed by a continuous decrease (significant ($P<0.05$) in Peldon) through to completion of the study. No significant difference ($P>0.05$) between the populations was observed with the exception of GSH in Peldon at GS26 being significantly higher ($P<0.05$) than Herbiseed. Within the individual populations GSH concentration was similar in plants under all of the regimes with similar responses.

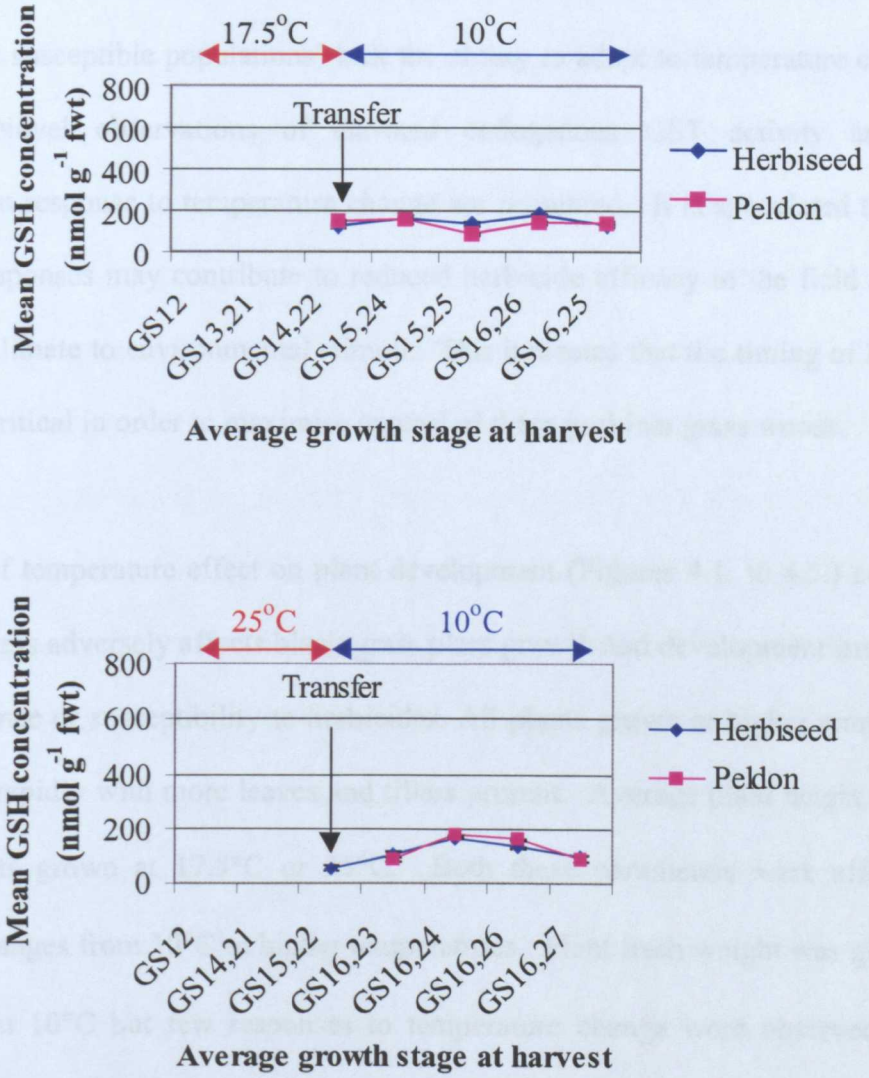


Figure 4.12. Mean GSH concentration (nmol g^{-1} fwt) of resistant and susceptible black-grass populations exposed to temperature transfers. GSH values are means ±SE values, where $n = 3$ plants. Error bars are included where they exceed the symbol size.

4.4. DISCUSSION

The objective of this study was to investigate the effect of temperature on the plant growth and foliar antioxidant status of resistant and susceptible black-grass populations. These findings indicate that temperature has a developmental and metabolic effect on resistant black-grass but susceptible populations' lack the ability to adapt to temperature change as efficiently. Novel observations of elevated endogenous GST activity and GSH concentration in response to temperature change are presented. It is speculated that these endogenous responses may contribute to reduced herbicide efficacy in the field as plants mature and acclimate to environmental stimuli. This indicates that the timing of herbicide application is critical in order to maximise control of these problem grass weeds.

Observations of temperature effect on plant development (Figures 4.1. to 4.5.) confirmed that thermal stress adversely affects black-grass plant growth and development irrespective of plant resistance or susceptibility to herbicides. All plants grown at higher temperatures matured more rapidly with more leaves and tillers present. Average plant height was also higher in plants grown at 17.5°C or 25°C. Both these parameters were affected by temperature changes from 10°C to higher temperatures. Plant fresh weight was greatest in plants grown at 10°C but few responses to temperature change were observed. Clear morphological effects were also exhibited. Apparently greater upright stance was observed in plants grown at 10°C or transferred to this temperature (Figures 4.1. – 4.5). Senescence was observed earlier (GS21/22) in plants grown at 25°C than those initially grown at 10°C (GS27/28). Temperature transfers from 10°C to higher temperatures induced senescence and wilting in some plants, as they were unable to immediately adapt to the change as exhibited in Figure 4.4. Transfers to 17.5°C induced immediate plant growth and development responses, whereas at 25°C they were observed later as a result of slower

plant acclimation. As all other growth conditions were unchanged for the duration of the experiments it is speculated that temperature was the key factor in inducing these effects.

Laboratory analyses revealed that black-grass grown at constant temperatures exhibits lower protein content and GSH concentration in combination with higher GST activity at 25°C. This confirms the findings reported in the previous chapter that increasing temperature is accompanied by a natural elevation in endogenous GST activity in resistant black-grass and it is known that black-grass is a temperate grass weed.

Analyses investigating the effect of temperature change revealed that temperature induced the activity and concentration of black-grass antioxidants in response to thermal stress. Response of resistant black-grass to temperature change triggered novel observations of changes in the concentrations and activity of the antioxidants GSH and GSTs. Large metabolic shifts in the form of elevated GST activity and GSH concentration when temperature was decreased from 25°C to 10°C, in Peldon confirm this. However, these elevations were transient and not maintained (Figures 4.7. – 4.10. and 4.12). It is postulated that even limited upward or downward shifts from normal growth temperatures which do not cause evident stress symptoms, might enhance the formation of AOS in black-grass. Both populations acclimated to initial growing temperatures as their growth up to GS12/13 was subsequently followed by a further 3 weeks at this temperature. However, the error bars surrounding the data points for GST specific activity of resistant black-grass post temperature change were large (Figure 4.8.) suggesting that plant acclimation to temperature did not occur at the same time in each individual plant, thus explaining the large degree of error and variation observed. This was not observed in Herbiseed, suggesting an inability to adapt to changing temperature as efficiently. These findings indicate that 25°C is a big penalty to black-grass over prolonged growth.

Distinction between plant resistance and susceptibility to herbicides was observed in the populations. As expected GST activity was consistently higher in Peldon but not always maintained (Figures 4.7. 4.8. and 4.10). The effect of changing and high temperature with respect to GST activity and resistance and susceptibility was significant, as activity of Herbiseed, despite variation, did not alter over time. In contrast, that of Peldon exhibited clear metabolic shifts (Figures 4.8. - 4.10). leading to resistance to susceptible ratios ranging between 1.0 and 3.0. being observed between the populations. The incidence of lower GST activity in susceptible black-grass suggests it does not adapt to changing temperature as efficiently as resistant plants, thus potentially rendering it more vulnerable to subsequent stress e.g. herbicide treatment. It is proposed that GST activity is inducible in susceptible Herbiseed in response to temperature, but is expressed constitutively in Peldon.

This investigation has looked at temperature extremes within a controlled environment as the cabinets used could only function at one temperature and were not programmable to introduce gradual temperature shifts such as those experienced in the field. Thus the stress responses observed here could mask the recognition of early and subliminal adjustment processes, which allow plant metabolism to gradually prepare for adverse thermal regimes in a field situation. The responses to the temperature transfers seen here could be termed as 'heat shock' responses although this was not explored. The growth cabinets used also limited the temperature range that could be examined particularly as these findings suggest that chilling could also invoke antioxidant response in black-grass weeds. However, the lowest temperature possible without large variation was 10°C, therefore this comparison in conjunction with time constraints was not possible.

The growth cabinets utilised during this study also had space limiting factors. In total, seven pots containing six plants of each population were grown for each temperature

regime, thus restricting observations and harvests to the end of tillering and vegetative development. This meant that only 3 plants of each population at each harvest were available for protein and GST analysis and the remaining 3 for GSH concentration determination. It would have been beneficial to monitor plant growth and antioxidant status well into reproductive growth and follow the populations' acclimation process over a prolonged period. However, the size of the growth cabinets dictated the number of pots used whilst ensuring plenty of space for plants to mature and develop under non-restricting conditions. In defence of this, with respect to herbicide application in the field, most herbicides for grass weed control are considered relatively ineffective post tillering; therefore these experiments could therefore be considered appropriate, although more plants for analysis would have been desirable. This point of discussion is also relevant to the variable coefficients of variation which were observed for these studies, as presented in Tables 4.2 – 4.5. Many of these figures were high as a result of practicality dictating the experiments. As few plants were available for analysis, it must be accepted that variation plays a large part in these experiments. Randomisation of the pots reduced bias within the experiment as much as possible by considering all growth cabinet features.

Laboratory methods adopted for protein content and GST activity analysis in this investigation are well-reviewed procedures carried out under standardised conditions, which are reproducible. Severe limitations were experienced with the GSH assay due to a requirement of a 1g sub-sample of tissue, which was unobtainable early in the experiments. In order to counteract this problem, more plants would be required for each population at the named growth stage / harvest point, but space restrictions in the growth cabinets would hamper this solution.

The incidence of biological activity in response to environmental stresses in plants is well-documented (Bartosz, 1997; Foyer and Fletcher, 2001). In particular it is well established

that high temperatures can have both direct inhibitory effects on vegetative growth and indirect effects, due to the high evaporative demand causing more intense water stress. Subjecting plants to moderately high temperatures such as the temperature transfers and 25°C constant experiments can cause plants to acclimate so that they can withstand higher temperatures (Hall, 2001). The results presented here support this theory.

Plants possess endogenous protective mechanisms known as antioxidants that scavenge and remove biologically active AOS that are generated in the chloroplast during photosynthesis (Halliwell, 1982; Halliwell and Gutteridge, 1989). It is well established that the photosynthetic system of plants can be affected by heat resulting in damage to components of photosytem II found in the thylakoid membranes of chloroplasts (Murukami, Tsuyama, Kobayashi, Kodama and Iba, 2000; Hall, 2001). Antioxidants are compounds capable of quenching AOS without themselves undergoing conversion to a destructive radical, but within every plant cell there is an important equilibrium between AOS formation and destruction. Any alterations in this equilibrium which lead to AOS accumulation are referred to as oxidative stress and AOS must be rapidly processed if oxidative damage is to be averted (Sies, 1991; Noctor and Foyer, 1998; Foyer and Fletcher, 2001). Oxidative stress halts normal metabolism and functions of plant cells resulting in a higher rate of accumulation of oxidative damage to cellular constituents. Cellular response may be adaptive through increased biosynthesis of antioxidant and AOS-decomposing enzymes as these findings confirm (Buttke and Sandstrom, 1994; Mostowska and Gwóźdź, 1995). However, it must be recognised that low concentrations of AOS are actively involved in plant development as they act as signals for growth, movement and differentiation as well as acclimatisation to changing environmental conditions (Sairam, Deshmukh and Shukla, 1997; Foyer and Fletcher, 2001).

During the acclimation process to different temperatures it has also been established that a

set of novel proteins are synthesised in plants known as 'heat-shock' proteins (Vierling, 1991). The heat shock response is a conserved reaction characterised by a rapid induction of the synthesis of heat shock proteins and acquisition of thermotolerance thus enabling cells to withstand and survive the detrimental effects of heat. Heat shock proteins are suggested to accumulate in a dosage-dependent manner in response to heat stress and are also induced by a number of other environmental stresses. These proteins may also be involved in providing cross-tolerance to some other stresses (Sabehat, Weiss and Lurie, 1998; Schöffl and Prändl, 1999). This is achieved by two mechanisms: as molecular chaperones and by targeting proteins for degradation (Lee and Vierling, 2000; Queitsch, Hong, Vierling and Lindquist, 2000). It must therefore be recognised that these proteins may have been present in the plants studied but their contribution to plant acclimation and herbicide resistance was not explored due to time limitations.

The many physiological and metabolic strategies adopted by plants to adapt to natural fluctuations in their thermal environment have long been and still are the subject of intense study (McKersie and Leshem, 1994; Badiani *et al.*, 1997). Species of plants vary greatly with respect to resistance to temperature. This investigation confirms the findings of Chauvel, Munier-Jolain, Letouzé and Grandgirard (2000) studying the developmental pattern of black-grass that temperature has a developmental effect on the growth of this species and may be critical in the response of plants to herbicide treatment. The fact that plants continuously modify aspects of their development to accommodate environmental change suggests the existence of sensors which monitor changes in the environment and signalling pathways which transduce this information to the appropriate responses of the cell cycle and plant defence mechanisms (May, Vernoux, Leaver, Van Montagu and Inzé, 1998; Knight and Knight, 2001).

Findings also presented here revealed that temperature change results in metabolic changes

in black-grass, many of which may be critical in the response of the plant to a herbicide. Many studies have indicated that the antioxidant systems of plants respond early and promptly to environmental stress (Scandalios, 1993; Foyer, Descourvières and Kunert, 1994b). The relationship between thermal environment and the thermal dependence and activity of enzymes is well established (Simon, 1979; Simon, Potvin and Blanchard, 1983; Selinioti, Manetas and Gavalas, 1986) but the extent to which the optimal thermal range for a plant can be altered by changes in activity of one or more enzymes is dependent upon which enzymes are affected by thermal stress (Mahan, McMichael and Wanjura, 1995). Survival of environmental fluctuation requires plant metabolism to be both flexible and dynamic as demonstrated by Peldon supporting the suggestion that modulation in the activities of antioxidants may be important in plant resistance to environmental stress. Exposure to one particular stress may also endow a plant resistance to subsequent stress e.g. herbicide treatment (Muzik, 1976; Tanaka and Sugahara, 1980; van Rensburg and Kruger, 1994; Sairam *et al.*, 1997; Foyer and Fletcher, 2001). However, in agreement with Sairam, Srivastava and Saxena (2000) plants respond differentially to various stresses as a result of variations in their antioxidant systems. This study revealed that temperature did not induce the metabolic changes in Herbiseed as seen in Peldon, which may contribute to its susceptibility to herbicides. However, it must be recognised that temperatures above the optimal range for plants do not always constitute a thermal stress, as the temperature of a plant is not always equal to the surrounding air (Mahan *et al.*, 1995).

The introduction of antioxidant enzymes has been well reported to be caused by and to take part in acclimation to both low (Schöner and Krause, 1990; Anderson *et al.*, 1992) and high (Rabinowitch and Fridovich, 1983; Kraus and Fletcher, 1994; Jagtap and Bhargava, 1995) temperature stress. The role of GSTs in endogenous metabolism is still largely unexplored but it is well established that specific GSTs accumulate in plants exposed to environmental stress which result in major shifts in metabolism (Marrs, 1996). This

suggests GSTs have roles in maintaining cellular homeostasis and counteracting stresses, particularly those as a result of oxidative stress (Dixon *et al.*, 1998; Edwards *et al.*, 2000). GST enhancement is now recognised as a marker for plant response to stress, although the functional significance of selective GST expression is only just emerging (Edwards *et al.*, 2000). Stress inducible GSTs might act to conjugate metabolites arising from oxidative stress. Phi GSTs from sorghum are known to detoxify 4-hydoryxnonenal released following oxidative membrane damage (Gronwald and Plaisance, 1998) and similar activities in tau GSTs of wheat have been observed (Cummins *et al.*, 1997b). Edwards *et al.*, (2000) suggest that GSH conjugation of electrophilic natural plant products through GST catalysation could have a large role. Tobacco seedlings overexpressing a tobacco tau GST with high GSH peroxidase activity are more tolerant of chilling than wild-type plants (Roxas *et al.*, 1997). It is postulated that GSTs present in resistant black-grass adopt a similar role which is not within susceptible plants ability. This is supported further by recent research, which has discovered a further link between GSTs functioning as GSH peroxidases and oxidative-stress tolerance in black-grass. Peroxidase activity has been shown in a number of inducible plant GSTs and suggests these enzymes play an important role in counteracting oxidative damage in plants arising from diverse biotic and abiotic stress (Edwards and Dixon, 2000). Herbicide resistant weeds which are cross-resistant to several classes of herbicides express a phi GST (*Am*GSTF1) that is a highly active GSH peroxidase, but which is barely detectable in herbicide-sensitive black-grass (Cummins *et al.*, 1999). It is speculated from the results of this investigation that these peroxidases may also function in tolerance against oxidative stress in response to adverse environmental conditions and is worthy of further study.

Insufficient data was acquired from this study to draw accurate conclusions about the effect of temperature on GSH concentration in black-grass. However, levels of GSH have been shown to correlate with the adaptation of plants to extremes of temperature (May *et*

al., 1998). Glutathione is an essential component of a plants defence system against environmental stress as GSH biosynthesis and accumulation are enhanced in response to plant stress as preliminary suggested by these findings (Rennenberg and Brunold, 1994; Foyer and Noctor, 2001). Oxidative stress can provoke large, but transient changes in the GSH: GSSG ratio leading to increased GSH biosynthesis, as demonstrated in Figure 4.12. (Smith, Kendall, Keys, Turner and Lea, 1984; Smith, 1985; Vanacker, Carver and Foyer, 2000). It is essential that sufficient amounts of reduced GSH and high GSH: GSSG ratios are present for GSH to fulfil its roles in metabolism and defence. Glutathione homeostasis regulation is affected by synthesis, degradation, transport and detoxification processes. If a plant is efficient, the GSH pool is low as it is utilised as soon as it is produced, maintaining equilibrium. When GSH acts as an antioxidant, it is oxidised to GSSG. Under unstressed conditions, GSSG is reduced efficiently back to GSH by the action of glutathione reductase (GR), such that the GSH pool is generally >95% reduced (Foyer and Rennenberg, 2000; Foyer, Theodoulou and Delrot, 2001). In extreme stress situations, the rate of GSH oxidation exceeds GSSG reduction, the GSH: GSSG ratio decreases, and this signals enhanced GSH accumulation (Noctor, Velijovic-Jovanovic and Foyer, 2000) as demonstrated in Figure 4.12. However, little is understood about the signalling mechanisms involved in induction of GSH synthesis. It cannot be assumed that the same regulatory factors operate under all conditions, but metabolic cross-talk enables GSH synthesis to respond to different environmental and metabolic triggers (Noctor and Foyer, 1998; Knight and Knight, 2001). It appears to be a universal response in plants faced by environmental stress where the antioxidant defences are temporarily overwhelmed by an oxidative burst or the accumulation of AOS as a result of impaired metabolism (Foyer and Rennenberg, 2000). The data presented here supports the literature, as GSH levels were clearly sensitive to thermal change.

The results presented here should also consider global warming with respect to herbicide

resistance and herbicide application. Predictions indicate that global climate change may increase the incidence of thermal stress as a result of changes in the ambient thermal environment and patterns of precipitation (Houghton, Meira Filho, Callander, Harris, Kattenberg and Maskell, 1996). Predictions suggest that by the 2050s, the UK will be 1-2°C warmer than at present, with winters predicted to warm more than summers. The novel observations presented here suggest that if the mean air temperature increases, antioxidant activity of black-grass may increase as plants acclimatise to the change in climate, which could consequently lead to more plants becoming resistant to herbicides. Even if plants are not resistant to the active ingredients of the future, these studies present elevated endogenous GST activity at high temperatures suggesting that plants may possess the ability to break down herbicides more quickly thus rendering them inactive. The de-registering of 70% of global active ingredients available to the agrochemical market by July 2003 will reduce the options farmers have to control these problem grass weeds. These arguments may or may not be valid, as it must be taken into consideration that measurements regarding the climate of the UK only began in the 17th century and therefore predictions are only based on 340 years of data. This does not take into account climate in previous centuries and thus these predictions must be treated with caution.

These findings confirm the prompt and perceptive nature of plant antioxidant systems as indicators of a changing growth environment (Scandalios, 1990; Hausladen and Alscher, 1993) but have raised areas worthy of further study. The main subject area to take this study further would be to investigate the factors that control and influence the GSH pool in black-grass and how it fits in with enzymes such as GSTs when responding to stress and its link to herbicide efficacy.

As discussed earlier, this investigation has looked at temperature extremes within a controlled environment. However, variations between plants can be caused by

unavoidable fluctuations in field conditions, coupled with diurnal and nocturnal changes in metabolic activity in the plants (Foyer, 1993; Hausladen and Alscher, 1993; Badiani, Schenone, Paolacci and Fumagalli, 1994). It could be speculated that the temperature transfers studied here induced 'heat shock' responses due to the large changes in temperature the plants were exposed to allowing the plants to acclimate to the changing temperatures. It is also well documented that the level of GSH correlates with the adaptation of plants to extremes of temperature (May *et al.*, 1998) and the levels of GST activity also respond to environment changes (Marrs, 1996). In the field situation, lower, more gradual temperature shifts would be observed than those studied here. It would be useful to analyse meteorological data and grow plants for a longer period e.g. two to three months with gradual temperature shifts both diurnal and nocturnal and observe any physiological and biochemical changes in the plants. Programmable environmental growth cabinets would permit this kind of study and allow more in depth interpretation of results by creating a more realistic environment when compared to a field situation to simulate day and night temperature shifts. This would allow monitoring of plants in the presence of both optimal and non-optimal growth temperatures that although may not be stressing, may act in nature as signals inducing specific paths of metabolic changes. This sort of investigation could present important opportunities for molecular study and could be taken further to investigation areas such as low temperature and water stress. Such molecular ecology comparisons could be carried out using black-grass as a model system for other grass weeds. Limitations of the cabinets used in this study meant that this kind of programme could not be carried out and only one set temperature could be monitored.

Other areas to investigate and develop are the speculated correlation between response to environmental stress and reduced herbicide efficacy. It is suggested plants are grown under similar temperature regimes as in these studies, but are also subjected to herbicide treatment and their responses monitored with respect to herbicide resistance. Additional

study is suggested in black-grass to investigate if a role exists for GSTs acting as GSH peroxidases with respect to oxidative stress tolerance as a result of adverse environmental conditions. Identification of the thermal range of black-grass would provide a means of quantifying thermal stress in these grass weeds and would allow predictions of any alterations in thermal dependence in the internal metabolism of black-grass and thus potentially could be used as a tool for predicting optimal herbicide application timings.

4.5. CONCLUSION

A series of experiments investigating the effect of temperature on plant growth and antioxidant status of untreated black-grass populations was carried out under controlled growth conditions. These findings indicate that temperature has a developmental and metabolic effect on the growth of resistant black-grass. Changing temperature has been shown to increase the concentration of antioxidants in the tissues of resistant black-grass thus making them resistant to subsequent stress. The incidence of lower concentrations in susceptible plants suggests that they do not adapt to changing temperature as efficiently as resistant plants, which in turn may render them more susceptible to herbicide treatment.

The antioxidant status of plants is vital for survival under normal environmental conditions. It is proposed that these endogenous responses are part of a normal mechanism of acclimation to environmental change in resistant black-grass. The consequence of such environmental plasticity through rapid phenotypic adjustment offers considerable advantage to resistant plants, allowing them to successfully survive environmental stress in response to changing season and thus in addition survive herbicide treatment. In striving to achieve maximum herbicide efficacy in resistant populations, the period of environmental change from autumn to winter as temperature decreases, in combination with smaller growth stages of plants, would be the best time for graminicide application for

black-grass control.

CHAPTER FIVE

GENERAL DISCUSSION

Herbicide resistance in black-grass has been thoroughly examined with respect to antioxidant concentration and activity and the potential links between GST activity and reduced herbicide efficacy have been investigated. These findings indicate that antioxidants form an integral part of a natural defence system in resistant black-grass plants against stress. It has been suggested that GSTs play a distinct role in enhanced metabolism resistance of black-grass to several graminicides through elevated activity and herbicide detoxification (Cummins *et al.*, 1997a; Reade *et al.*, 1997; Edwards and Dixon, 2000). The work presented here supports this theory, but also indicates that GSTs and GSH and their role in endogenous metabolism is equally important.

The following points summarise the main findings of this thesis:

- The Herbiseed population may be used as a standard susceptible population when testing unknown populations.
- The antioxidant status of black-grass plants is vital for survival under normal environmental conditions.
- Endogenous antioxidant responses form part of a natural mechanism of acclimation to environmental change in resistant black-grass and render them resistant to subsequent stresses such as herbicide treatment.
- Natural elevations of endogenous GST activity normally occur during plant growth of black-grass.
- GSTs are required as part of the black-grass antioxidant defence system to protect developing plants from toxic endogenous substrates associated with environmental stress and possess direct cytoprotective activity during environmental changes and stress in conjunction with supporting normal plant development.
- Temperature has a developmental and metabolic effect on the growth of resistant black-grass.

- Susceptible black-grass does not adapt to changing temperature as efficiently as resistant black-grass, rendering it more susceptible to herbicide treatment.
- These findings lend further weight to the suggestion that the development of resistance in black-grass is in part due to evolution and elevation of GST activity.
- GST activity could potentially be used as a marker for resistance in the field and for predicting herbicide application timings.
- To effectively control black-grass populations, both resistant and susceptible, herbicide application must be early in the growth season during the period of environmental change from autumn to winter to ensure maximal herbicide efficacy.

Scientific investigations are carried out under specific experimental conditions using several different pieces of sometimes specialist equipment. Different experimental procedures or use of different equipment could generate results of a different nature. There is a requirement to consider the appropriateness of the techniques used as the conclusions presented here do not exist in isolation from other studies of herbicide resistance in black-grass.

These investigations have utilised plants grown in controlled environment cabinets, the glasshouse and in the field. There are obvious dangers of extrapolating observations from controlled environments to the field as there can be large discrepancies when trying to compare data between what are three very different situations. This thesis concentrated on herbicide resistance and it is speculated that the resistance factor causing variability in plant responses has a far greater role to play when considering the results from the controlled environment cabinets, glasshouse and field (Krähmer and Russell, 1994). Plants grown and harvested from the field were exposed to the complex interactions of individual plant physiology, physical properties of the surrounding soil and the influence of the environment and climatic factors i.e. all normal processes were in place. Thus,

theoretically, they provided the best system with which to study growth and development of black-grass with respect to antioxidant activity. It must also be recognised that the populations grown in controlled environments for the purpose of this thesis were characterised whereas those in the field were unknown and contained a mixture of resistant and susceptible individuals. Plants grown under controlled conditions are normally subjected to two or three levels of one climate parameter while other parameters are kept constant as in Chapter 4. These environments are suitable when studying the influence of individual climate parameters, but are not exempt of problems.

A major limitation of the growth cabinets used for investigation in Chapter 4 was that only temperature extremes were studied since they could only function at one temperature and were not programmable to introduce gradual temperature shifts such as those experienced in the field. Hence the stress responses observed here could mask the recognition of early and subliminal adjustment processes, which allow plant metabolism to gradually prepare for adverse thermal regimes in a field situation. The responses observed here could be termed as 'heat shock' responses, but this was not explored as discussed in Chapter 4. The growth cabinets used, also limited the temperature range that could be examined, as the lowest temperature possible without large variation was 10°C. The growth cabinets utilised during this thesis were also space restricting, limiting the number of pots and plants being grown without restricting plant development. Thus plant sampling and observations were only carried out to the end of tillering and vegetative development as no more plants could be fitted in to allow them to progress into the reproductive stages. Limited space was also a problem in the glasshouse with respect to the investigation presented in Chapter 2. The co-efficient of variation (18.1%), although acceptable, could have been lower if more replicates had been introduced into the experiment. However, both space and time limitations were restricting.

The response of plants varies with growth parameters and experimental procedures. As a result, it is concluded that general transfer factors cannot be created for glasshouse/controlled environment to field transfer (Krähmer and Russell, 1994). However, if resistant and susceptible populations are grown alongside each other simultaneously as throughout this work, variation should be minimised. Seed germination under controlled environments was synchronised so plants were at the same developmental stage at time of spraying or harvest, but this is not true of the field. Extrapolation of results to the field can be difficult as selectivity of compounds in the field depends upon several factors (timing of application, growth stage of the weeds, climate conditions and soil characteristics) whereas controlled environments, water regimes, pot size and competition in the greenhouse are not representative of the field. Plants deemed resistant in the field may actually be susceptible under glasshouse/controlled environment conditions or at different geographical sites (Devine *et al.*, 1993). Factors such as high temperatures in the glasshouse as experienced in Chapter 2 and regular watering of plants in controlled environments result in atypical soil flora. The addition of artificial light as used in these studies differs from natural light and is usually of a lower intensity leading to plants which are morphologically and physiologically different from field grown plants. The lack of natural fluctuations in temperature and humidity will also contribute to this (Devine, 1988; Garrod, 1989; Bartley, 1993; Krähmer and Russell, 1994). This makes the data in this thesis important as both field and controlled environment data is presented. Plants grown under controlled conditions also experienced restricted root volume and were provided with a non-limiting water supply to counteract moisture stress. In the field, plants typically experience periods of at least mild moisture stress, which affects leaf and cuticular development and growth of primary roots (Davies and Blackman, 1989). Although controlled environments allow for a ranking of climate parameters, their relevance to the more complex field situation where factors fluctuate and interact is questionable. This would have been a factor to consider if a standard susceptible population was grown in the

glasshouse for sampling during the investigation presented in Chapter 3. Comparison to the field data would have been very difficult.

Field studies, although valuable, can make investigations labour intensive. The field studies presented in Chapter 3 incorporated extensive travelling and field sampling was very time consuming. This was problematic as the investigation in Chapter 3 would have benefited from the inclusion of additional sites located in a wider geographical area. This would have enabled influencing factors such as location, soil type and environment to be more widely studied. The investigation in Chapter 3 would have also benefited from the inclusion of black-grass plants from a standard susceptible population and from herbicide treated plots for comparison. However, it would have been impossible to identify a completely susceptible field black-grass situation as most populations are made up of both resistant and susceptible individuals. In hindsight, growing a susceptible standard such as Herbiseed in a glasshouse may have sufficed, but extrapolating the results to the field for comparison would have been difficult due to very different growth conditions. Plants sampled from treated plots would have been valuable to this study to compare the induction of GST activity by herbicide treatment with that activity which is naturally exhibited by black-grass plants as a result of changing plant growth and development and environmental conditions. However, time limitations with respect to travelling, sampling and laboratory analysis, plus space limitations with respect to storing samples did not permit an extensive study of this nature.

Plant sampling and its accuracy of representing the whole weed population of a site is a topic of great debate. Samples from field plots are small portions of a population taken for detailed study and as in Chapter 3 are composed of both resistant and susceptible individuals (Beckie *et al.*, 2000; Clewer and Scarisbrick, 2001). The system adopted for this thesis relied on regular descriptive samples, which allowed environmental effects on

plant morphology and metabolism to be analysed whilst being representative of the population. Heap (1994) suggests that 40 plants should be sampled from field weed patches but time and space limitations did not permit this. Careful assessment of plant establishment within plots prior to initial sampling was carried out. Other limitations were that sampling for this investigation was limited to a commercial trial site as opposed to access to the entire field. A subsampling system whereby the plant in the bottom left hand corner of the quadrat was sampled was adopted to eliminate bias between large and small plants. Limitations of sampling include the wide range of plant-to-plant variation, which if only small numbers of plants are studied, cannot be quantified accurately (Clewer and Scarisbrick, 2001). Samples taken from the glasshouse or controlled environment cabinets meant that all the plants from a particular pot were harvested at each time point and thus these arguments are not applicable.

The work of this thesis was dependent upon the provision and use of whole plants although GST activity can be determined in all plant parts (Edwards and Dixon, 2000). The advantage of whole plants as opposed to isolated plant organs is that they are what most closely resemble the field situation. The use of whole plants in Chapter 2 gave good indications of herbicide action in the field as correlation between the field and glasshouse results could be made. They have an advantage over the use of single leaves or isolated organs as all normal plant processes are in place for the uptake, regulation and translocation of the herbicide (Clarke and Moss, 1991). However, it must be recognised that this advantage is balanced against the contribution of individual processes, which cannot be quantified. Initial characterisation of herbicide resistance still relies on detailed dose response experiments in pot-experiments assessing the whole-plant response (Heap, 1994), which justifies their use throughout this thesis. This is further supported by statistical analysis carried out on individual leaf responses prompting data to be presented on a whole-plant basis as described in Chapter 3. Substantial herbicide research

particularly studying enzymes is carried out using cell and tissue cultures, as they are less time-consuming, labour intensive and space requiring then growing pots of plants. Herbicides easily enter cells and interact with target sites and enzymes involved in herbicide metabolism. Limitations of these methods include that there can be no differentiation and no chlorophyll is present and thus photosynthesis is absent and hence metabolism observed in cell cultures may not represent that of whole plants (Cobb, 1992). The practicality of these approaches depends upon the results required in conjunction with resources available. The use of whole plants for this study was appropriate for comparison to field situations, but space limitations within the glasshouse and controlled environment cabinets meant that large-scale experiments were not possible. For example, more plants would be necessary to generate more fresh weight material for analysis or the inclusion of a susceptible standard suggested in Chapter 3. In addition, due to the time taken to conduct laboratory analyses these additional factors would not have been feasible to include in the investigations.

All extraction and analyses were carried out under standardised and reproducible conditions. GST activity was determined *in vitro* using the standard experimental assay using the artificial substrate CDNB and was developed into simple microplate assay. The initial limitation of this assay was that CDNB is not a model substrate for all GSTs as some GSTs show negligible activity or are undetected when using this assay and that measurements *in vitro* may not correlate to the ability of the plant to form GSH conjugates *in vivo* (Marrs, 1996; Cole and Edwards, 2000). The GST activities presented here using CDNB may be a lot higher than if endogenous substrates such as the herbicides applied in Chapter 2 had been used (Gronwald, Fuerst, Eberlein and Egli, 1987). CDNB produced higher rates of GST activity, which facilitates detection, but may have reduced measurable differences between the populations studied. This assay can also not be used to identify differing isozymes, or quantify the amounts, which are present. The use of CDNB as a

substrate probably underestimated the true activity of GSTs within individual plants and leaves in this thesis and hence, there could be differences between the populations, which were not detected. The microplate assay utilised here was an effective method of determining GST activity. Other methods for GST determination include a versatile using high-performance liquid chromatography (HPLC) based assay system, which was developed to assess the reactivity of agrochemicals with GSH, with and without catalysis by GSTs (Clarke *et al.*, 1998). Due to time restraints it was not possible to adopt this method. In defence, the microplate assay was simple to prepare and carry out, allowing numerous samples to be processed at one time. This provides considerable advantages to laboratories wishing to gain further insight into the role of both GSH and GST mediated metabolism. Another limitation of the GST assay was the time taken to prepare cell-free extracts for microplate analysis. At most only 50 samples could be comfortably prepared in a day, which limited the amount of plant material grown for experiments and number of samples taken during the investigations.

Numerous procedures have been developed for the determination of GSH concentration using a variety of detection systems. Currently most determination is carried out by HPLC, but time restraints meant it was not possible to learn and develop this technique. However, it must be recognised that the methods used for the determination of GSH in black-grass in this thesis were adapted for black-grass from the cell-free extracts method of Griffith (1980) and the microplate method as described by Baker *et al.*, 1990. Both methods had to be adapted on a trial and error basis in order that they were appropriate for use with black-grass. Preparation of cell-free extracts required significant time to establish (using specially grown plant material) the correct amount of fresh black-grass tissue to use plus the requirement to identify how much homogenisation or centrifugation was required to produce a suitable supernatant for further analysis. The microplate method required a substantial amount of time in order to determine correct solution molarities, the relative

concentrations in the reaction mixture and appropriate GSH stock solutions to determine a standard curve appropriate to black-grass. The methods were tried and tested a number of times before being used for samples grown in experiments.

Separate plant material was required to carry out the GSH assay, which inevitably created a larger workload. This was because the crude protein extracts used for GST determination were desalted through Sephadex PD10 columns and GSH, being a small molecule was eluted and lost during this process, hence the requirement for extra plant material. The adapted extraction method was suitable but required a 1g sub-sample of tissue, which was unobtainable early in the investigations due to small plants. The microplate assay was also adapted and developed for the purpose of this thesis with the advantage of the inherent ability to assay a large number of samples simultaneously. The accuracy of the assay largely depends upon the accuracy in sample processing and pipetting. Assay limitations included the standard curve, which only covered 100-400 pmol as readings containing lower GSH standard concentrations were inaccurate and unreliable and the standard curve was no longer linear. This affected readings of low sub-samples of tissue, which were found to be unreliable and unusable resulting in many observations not being obtained for as many as the first 4 harvest points as demonstrated in Chapter 4. This invariably restricted conclusions, which could be drawn with respect to GSH concentration and herbicide resistance. In order to counteract this problem, more plants would be required for each population at the named growth stage / harvest point in order to obtain the required sub-sample. However, space restrictions in controlled environment cabinets would hamper this solution. Other limitations were that due to the potential rapid loss or change in oxidation state of intracellular GSH, all tissue samples had be processed as quickly as possible i.e. immediately post extraction (Baker *et al.*, 1990) which required time organisation. Freezing extraction tissue samples was not a viable

option, as in practice runs, GSH levels were found to be either undetectable or very unreliable once samples were thawed.

All data presented here is expressed on a fresh weight and protein basis. When drawing conclusions, it is more appropriate to consider data expressed on a protein basis as it is more accurate to correlate GST activity to the protein content of the extracts. However, activity or concentration of antioxidants is easier to calculate on a fresh weight basis and goes some way to allowing results to be compared with those from the field. As discussed in Chapter 3, both methods were used to determine GST activity to allow conclusions to be drawn by comparing and contrasting GST activity at two levels. Specific activity determined whether GST activity was changing at a cellular level as a result of changes in GST protein or activity, or if indeed other factors such as plant dehydration were playing a role (GST activity g^{-1} fwt).

Plants have evolved a wide range of defence systems, both constitutive and inducible, to survive the continuous assault of both abiotic and biotic stress. This thesis has examined one small part of this defence system and how it is employed against active oxygen species as a result of abiotic and biotic stress, and how it may contribute to black-grass becoming resistant to herbicides. The findings of this thesis in Chapter 2 and Appendix 1 highlighted abiotic stress through herbicide treatment and confirmed that it induces GST activity particularly in resistant black-grass. Chapters 3 and 4 investigated the natural roles of GSTs and GSH within the plant with respect to biotic stress and herbicide resistance as a result of different factors such as plant growth and development, constantly changing weather or other environmental stresses. Figure 5.1. illustrates the proposed roles of GSTs and GSH in black-grass with respect to defence against abiotic and biotic stress and resulting AOS, through detoxification and induction. Figure 5.1. also places both GSTs and GSH into context with respect to induction, detoxification and metabolism carried out

by other enzymatic and antioxidative systems speculating how they may operate in a whole plant when subjected to biotic stress. However, it must be recognised that many other factors can lead to reduced herbicide efficacy such as the growth stage of the weed, season, environmental conditions at the time of spraying and soil characteristics.

The findings of this thesis agree with the literature that GSTs play a role in herbicide metabolism and detoxification (Chapter 2 and Appendix 1) and the defence system process as discussed in Chapter 1 is summarised in Figure 5.1. (Edwards, 1997; Edwards and Dixon, 2000). Many of the enzymes involved in the detoxification and metabolism of herbicides and the biosynthesis and transport of herbicide conjugates are developmentally regulated. Thus, they are also induced or enhanced by biotic stress such as environmental factors, infection and other abiotic stress (Cole and Edwards, 2000) as indicated by GSH and GSTs in this thesis. However, the full roles of GSTs and GSH remain largely unexplored. This work implies that they are an essential part of the antioxidant defence system of black-grass against biotic stress allowing plants to acclimate and adapt to their surrounding environment. The findings of this thesis support the suggestion that certain enzymes involved in the detoxification of herbicides are also involved in protecting plants from toxic natural products parallel to their action in detoxifying xenobiotics (Cole and Edwards, 2000).

Enzymes involved in natural product metabolism in plants are subject to complex regulation during the life cycle of a plant (Holton and Cornish, 1995). However, few studies have been carried out which determine developmental influences on enzymes involved in herbicide metabolism. GSTs are well established to be developmentally regulated (Cole and Edwards, 2000) as confirmed by the findings of Chapter 3. Molecular studies investigating regulation by biotic stress indicate that messenger RNAs encoding GSTs accumulate in plants exposed to heat-shock such as that imposed in Chapter 4 and

other biotic stresses as well as treatment with plant hormones and other natural products (Marrs, 1996). However, with a few exceptions, effects on herbicide conjugation have not been studied as the enzymes encoded by the inducible transcripts have not been characterised (Cole and Edwards, 2000). Of particular interest to this thesis is the evidence that in soybean, two distinct GSTs have been found to be inducible by heat-shock and auxins, and both have activity towards herbicides (Flury, Adam and Kreuz 1995; Andrews *et al.*, 1997; Skipsey, Andrews, Townson, Jepson and Edwards, 1997).

The potential for oxidative stress is constant and inescapable, therefore efficient antioxidant defences such as GSH and GSTs are essential for plant acclimation and adaptability to the surrounding environment. It is postulated from these findings that normal plant development and environmental change such as those investigated in Chapters 3 and 4 induce over-production of AOS, thus upsetting the equilibrium between AOS production and degradation (Figure 5.1). It is speculated that GSH and GSTs form part of the defence system against AOS endowing metabolic plasticity, subsequently enabling the plant to adapt and acclimate to changes in its environment such as those observed in Chapter 3 and induced in Chapter 4. These processes are postulated to form Phases 2 and 3 of the defence process against biotic stress (Figure 5.1). Increased understanding of the molecular basis of the plasticity of plant defence metabolism could provide farmers of the future with more crop management tools with which to control herbicide resistant grass weeds such as black-grass without increased reliance on chemical control means.

The investigations of this thesis have concentrated on the roles that GSTs and GSH play in herbicide resistance in black-grass and have highlighted their importance in detoxification and induction as a result of both abiotic and biotic stress. However, P450s are recognised as the most important enzyme system for Phase 1 metabolism of herbicides as they are able

to utilise herbicides as substrates in plants and it may be that they also have roles in the defence against biotic stress prior to the involvement of GSH and GSTs (Figure 5.1). The regulation of P450 activities and endogenous metabolism in plants is poorly understood. P450 activity, like GSTs can be constitutive or induced by natural or xenobiotic stress (Frear, 1995). In particular, P450s, like GSTs have natural roles in endogenous metabolism. Their diversity is related to the roles that they take in secondary metabolism and plants use them to generate a vast array of defence compounds through the biosynthesis of structural compounds e.g. flavonoids. The majority of these act as precursors of signalling molecules that are important in plant defence such as pathogen attack to adverse environmental conditions (Durst and O'Keefe, 1995; Schuler, 1996; Chapple, 1998; Barrett, 2000). Therefore, it could be that P450s generate compounds or molecules, which act to induce GSH and GSTs as protection against oxidative stress.

Together, P450s and GSTs are regarded as the two primary enzyme systems acting to counteract both natural and xenobiotic stress affecting plant (Frear, 1995; Barrett, 2000; Devine and Preston, 2000). In resistant weed species e.g. black-grass, it is well documented that one or more phases of the herbicide metabolism process may be increased to aid herbicide detoxification, hence the speculation that this may be case with respect to natural biotic stress. Detection of P450s is achieved through microsomal assays, but although useful they are limited, in that total characterisation of the metabolism reaction cannot be achieved. As a result, the relative ease with which GSTs can be extracted and studied means that the understanding and molecular biology of GSTs is much more advanced that that of P450s (Marrs, 1996). The role of P450s in the endogenous metabolism of black-grass is relatively unknown, and was not explored during this research.

It is postulated that on induction by stress signallers or markers, non-enzymatic antioxidants (e.g. GSH) act as a first line of defence without the intervention of antioxidant enzymes when oxidative stress is initially detected within the plant. This suggests that the responses to small changes are an initial step in an efficient adaptive strategy aimed at preserving resources in the presence of moderate metabolic changes and at producing the maximal compensative effort only if and when needed. However, if oxidative stress increases and is prolonged there is a requirement for more energetically expensive responses such as the induction of scavenging and antioxidant-regenerating enzymes such as GSTs to protect the plant. These responses lead to the event of oxidative stress being transient and the plant adapts and recovers. However, if oxidative stress intensifies, the natural defence system of the plant will be overwhelmed and the effects irreversible, leading to plant death.

It is clear that active herbicide detoxification is a result of induced P450 and GST activity within black-grass and wheat (Kemp *et al*, 1990; Jablonkai and Hatzios, 1991; Tal *et al.*, 1993; Hall *et al*, 1995; Koeppe *et al.*, 1998). The induction and ability of GSTs to detoxify herbicides is not discounted, but the presence of elevated activity in resistant plants when sprayed in the field may not be totally due to stress from herbicide treatment as postulated here. This is supported by the observation of black-grass populations surviving herbicide treatment consisting of plants with higher GST activities, even with respect to herbicides that are not metabolised by this enzyme family (Reade *et al.*, 1999). It may be that GST isozymes induced by normal plant growth and development at the normal time of spray applications for the control of black-grass are present in conjunction with those which detoxify herbicides or they possess the ability to be induced both by abiotic and biotic stress. The presence of these isozymes would contribute to the individual plants resistance to the herbicide applied as a result of defence against subsequent stress, but this requires further study for validation. However, it is also clear that GSTs only form part of the

black-grass antioxidant system as opposed to acting in isolation, thus suggesting that there may be additional contributory factors present within this system towards herbicide resistance such as that provided by P450s. This provides a solid basis for future studies on herbicide resistance in black-grass.

The work of this thesis has highlighted the role of the black-grass antioxidant system in response to oxidative stress associated with normal physiology and climate change. The natural progression from this work would be to investigate the factors that control and influence the GSH pool in black-grass and other problem grasses, which would be of paramount interest with respect to herbicide resistance and herbicide application timing. In addition, investigation into the role and activities of a variety of other enzymes, in the endogenous metabolism of grass species and are active in the detoxification of AOS in response to stress such as superoxide dismutase, would be beneficial. Figure 5.1. illustrates that GSH and GSTs represent only part of several detoxification systems making up the natural defence system of black-grass. The relevance of this work to other grasses would also be important as throughout the UK, wild oats and Italian rye-grass are considered problematic grass weeds alongside black-grass.

The relevance of this work to these grass species where herbicide resistance is also a problem may go some way to their control. Preliminary studies on rigid rye-grass were carried out alongside black-grass with respect to the effect of temperature of plant growth and antioxidant status. However, the findings indicated that temperature change did not induce the developmental and metabolic effects seen in black-grass. Many other detoxification systems consisting of non-enzymatic and enzymatic components which detoxify AOS are well established but their roles in grass weeds remain to be identified and characterised. Further understanding of the relationship between detoxification

systems and their endogenous substrates within grass weeds and how they react to environmental stress would be of considerable benefit to farmers in the future as few novel weed control options are at their disposal.

In addition to this, further work which isolates and identifies GSTs isozymes as constitutive or inducible in grasses whilst also investigating their activity spectrum as so few GST isozymes have been fully characterised would be beneficial. This should be conducted both in a field situation and programmable environmental growth cabinets. Preliminary studies on the effect of S concentration on black-grass growth and development were also carried out. Although initial findings indicated that GSTs were not involved in black-grass defence against nutrient stress, it was clear that S was essential for black-grass growth and development. Further study, which identifies the role S plays in black-grass with respect to antioxidant systems and identifies any link with herbicide resistance, would be beneficial particularly as the UK moves into a period where S deficiency is becoming increasingly important with respect to the growth of cereals. This work could be achieved through hydroponic study assessing the role of S in black-grass with respect to GSH and other potential antioxidant systems. Assessment of the amino acid pool present in plants grown under optimum and deficient S conditions would also be beneficial as S is an essential constituent of the essential amino acids cysteine and methionine.

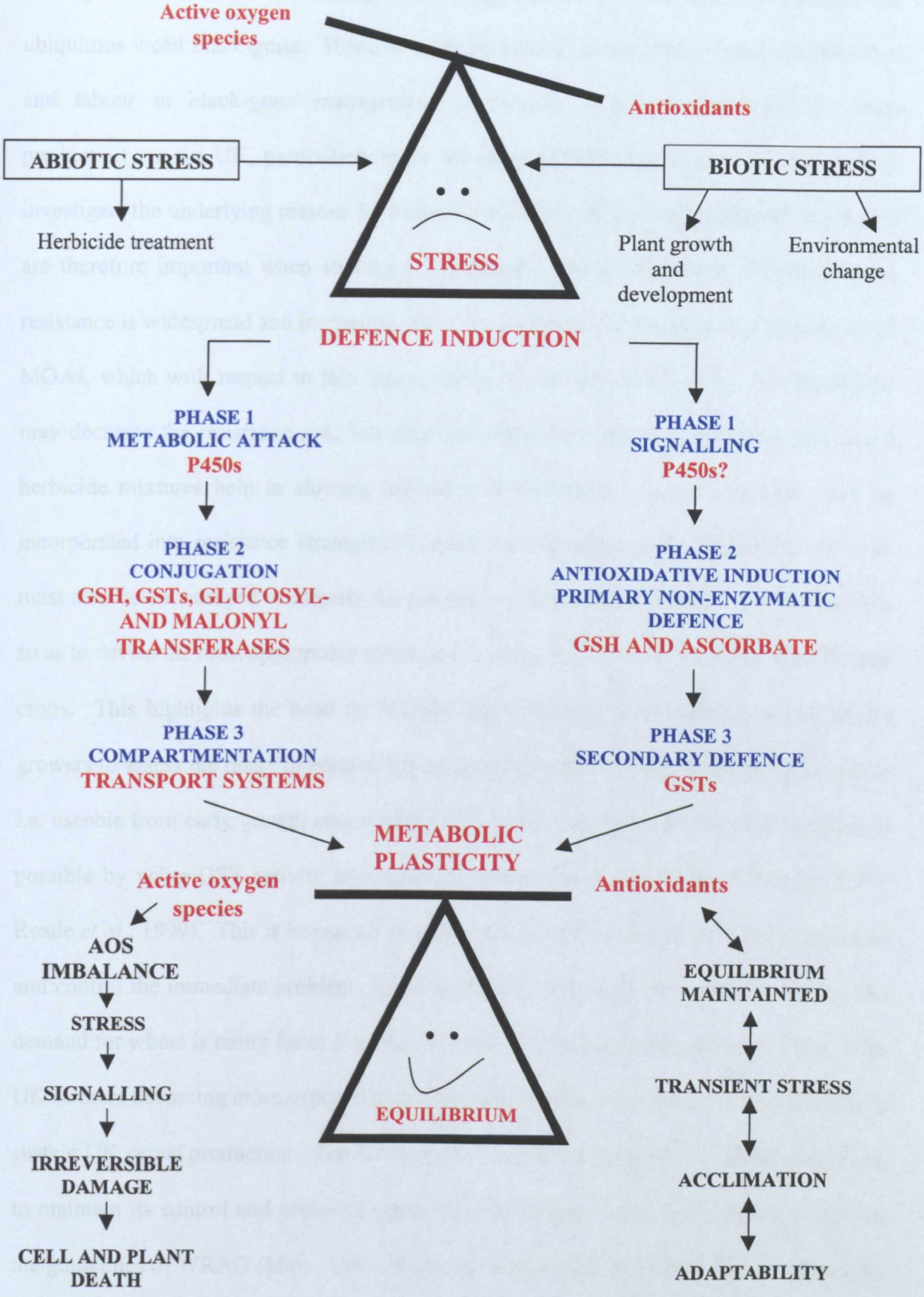


Figure 5.1. Schematic diagram showing the proposed roles of glutathione *S*-transferases and glutathione in defence against abiotic and biotic stress (see text for details).

Throughout the UK, farmers manage cereal cropping systems to reduce the impact of the ubiquitous weed black-grass. However, despite annual investment of financial resources and labour in black-grass management, it remains a serious constraint to cereal productivity in the UK, particularly since the onset of herbicide resistance. Research to investigate the underlying reasons for reduced herbicide efficacy and guidelines to avoid it are therefore important when striving for maximum yields and profits. As black-grass resistance is widespread and increasing, there is an emphasis to discover and develop novel MOAs, which with respect to this thesis, GSTs are not able to detoxify. Mixing MOAs may decrease the resistance risk, but does not eliminate it and it is debatable how much herbicide mixtures help in slowing resistance development. Cultural controls must be incorporated into resistance strategies to avoid over-dependency on herbicides. Farmers must also be encouraged to identify the resistance mechanism present as early as possible so as to devise the most appropriate resistance strategy, not just in cereals, but also in break crops. This highlights the need for a rapid diagnostic test for resistance, which allows growers to assess the range of control options available early in the current growing season i.e. useable from early growth stages of the weed which the basis of this work suggests is possible by using GST activity as a marker (Milner, Reade and Cobb, 1999 and 2001; Reade *et al.*, 1999). This is because it is of utmost importance that farmers not only assess and control the immediate problem, but also consider the longer-term consequences. The demand for wheat is rising faster than the growth in world population (Orson, 1999). With UK farmers becoming more exposed to international markets, every effort must be made to sustain UK cereal production. The battle against resistant black-grass is continuous and so to maintain its control and prevent resistance development, farmers are advised to follow the guidelines of WRAG (Moss, 1997) whilst the agricultural industry looks for innovative solutions to its diagnosis and control. The work described in this thesis has gone part way to providing a clearer picture of the underlying reasons for herbicide resistance in black-grass and the role of GSTs in endogenous metabolism. This has been achieved by the

development and adaptation of novel methodology for use in black-grass. The findings of this thesis have provided a valid basis for future work, which would undoubtedly contribute to how farmers prevent and manage herbicide resistant black-grass in the future.

CHAPTER SIX

REFERENCES

6.0 REFERENCES

AgrEvo (1997a). *Weed Guide.* King's Lynn: AgrEvo UK Crop Protection Limited.

AgrEvo (1997b). *Practical grass-weed management.* King's Lynn: AgrEvo UK Ltd.

AgrEvo (1998). *Cheetah Super Product Label.* King's Lynn: AgrEvo UK Limited.

Alscher R G (1989). Biosynthesis and antioxidant function of glutathione in plants. *Physiologia Plantarum*, **77**, 457-464.

Alscher R G, Donahue J L, and Cramer C L (1997). Reactive oxygen species and antioxidants: relationships in green cells. *Physiologia Plantarum*, **100**, 224-233.

Anderson M P and Gronwald J W (1991). Atrazine resistance in a velvetleaf (*Abutilon theophrasti*) biotype due to enhanced glutathione *S*-transferase activity. *Plant Physiology*, **96**, 104-109.

Anderson J V, Chevone B I and Hess J L (1992). Seasonal variation in the antioxidant system of eastern white pine needles. Evidence for thermal dependence. *Plant Physiology* **98**, 501-508.

Andrews C J, Skipsey M, Townson J K, Morris C, Jepson I and Edwards R (1997). Glutathione transferase activities toward herbicides used selectively in soybean. *Pesticide Science*, **51**, 213-222.

Appleby A P, Valverde B E (1989). Behaviour of dinitroaniline herbicides in plants. *Weed Technology*, **3**, 198-206.

Archer J (1988). *ed. Crop nutrition and fertiliser use.* 2nd Edition. Ipswich: Farming Press Ltd.

Avery D T (1995). *Saving the planet with pesticides and plastic: The environmental triumph of high-yield farming.* Hudson Institute, Indianapolis, Indiana, USA.

Ayres P and Smith P G (1985). The use of low doses of isoproturon, with and without the addition of a mixture of ioxynil and bromoxynil, for the control of *Alopecurus myosuroides* in winter cereals. *Aspects of Applied Biology – The biology and control of weeds in cereals*, **9**, 111-116.

Badiani M, Schenone G, Paolacci A R and Fumagalli I (1994). Daily fluctuations of antioxidants in bean (*Phaseolus vulgaris* L.) leaves. The influence of climatic factors. *Agrochimica* **38**, 25-36.

Badiani M, Paolacci A R, Miglietta F, Kimball B A, Pinter Jr P J, Garcia R L, Hunsaker D J, LaMorte R L and Wall G W (1996). Seasonal variations of antioxidants in wheat (*Tricitcum aestivum*) leaves grown under field conditions. *Australian Journal of Plant Physiology* **23**, 687-698.

Badiani M, Paolacci A R, Fusari A, D'Ovidio R, Scandalios J G, Porceddu E and Sermanni G G (1997). Non-optimal growth temperatures and antioxidants in the leaves of *Sorghum bicolor* (L.) Moench. II. Short-term acclimation. *Journal of Plant Physiology*, **151**, 409-421.

Baker M A, Cerniglia G J, and Zaman A (1990). Microtiter plate assay for the measurement of glutathione and glutathione disulfide in large numbers of biological samples. *Analytical Biochemistry*, **190**, 360-365.

Baldwin J H (1979). The chemical control of wild oats and black-grass. *ADAS Quarterly Review*, 33, 69-101.

Baldwin J H (1981). The chemical control of wild oats and black-grass. A review based on results from ADAS agronomy trials. *ADAS Quarterly Review*. London: HMSO. p. 69-94.

Barralis G (1968). Ecology of black-grass (*Alopecurus myosuroides* Huds.). *Proceedings of The Ninth British Weed Control Conference, 1968*, **1**, 6-8.

Barrett M (1995). Metabolism of herbicides by cytochrome P450 in corn. *Drug Metabolism and Drug Interactions*, **12**, 299-316.

Barrett M (2000). The role of cytochrome P450 enzymes for herbicide metabolism and selectivity. **In: Cobb A H and Kirkwood R C**, *eds. Herbicides and their Mechanisms of Action*. Sheffield: Sheffield Academic Press. p. 25-37.

Bartley M R (1993). Assessment of herbicide selectivity. **In: Streibig J C and Kudsk P** *eds. Herbicide Bioassays*. Boca Raton, Florida: CRC Press. pp. 57-73.

Bartosz G (1997). Oxidative stress in plants. *Physiologiae Plantarum*, **19**, 47-64.

Beckie H J, Friesen L F, Nawolsky K M and Morrison I N (1990). A rapid bioassay to detect trifluralin-resistant green foxtail (*Setaria viridis*). *Weed Technology*, **4**, 505-508.

Beckie H J, Heap I M, Smeda R J and Hall L M (2000). Screening for herbicide resistance in weeds. *Weed Technology*, **14**, 428-445.

Behrendt S and Hanf M (1979). *Grass weeds in world agriculture.* Ludwigshafen: BASF.

Blair A M (1978). Some studies on the sites of uptake of chlorotoluron, isoproturon and metoxuron by wheat, *Avena fatua* and *Alopecurus myosuroides*. *Weed Research*, **18**, 381-387.

Blair A M (1985). Influence of soil moisture on isoproturon activity against *Alopecurus myosuroides*. *Weed Research*, **25**, 141-149.

Blair A M (1986). The effect of shading on isoproturon activity on *Alopecurus myosuroides*. *Tests of Agrochemicals and Cultivars*, **7**, (*Annals of Applied Biology, **108**, Supplement)*, 100-101.

Blair A M and Orson J H (1993). Evaluation of multi-herbicide mixtures against *Alopecurus myosuroides* (black-grass) in winter wheat. *Tests of Agrochemicals and Cultivars – Annals of Applied Biology (Supplement)*, **14** (122), 80-81.

Blair A M, Clarke J H and Orson J H (1996). Effects of soil type, weather and resistance on efficacy of herbicides against black-grass. *HGCA Project Report No.131.* London: HGCA.

Boutsalis P (2001). Syngenta Quick-Test: A rapid whole-plant test for herbicide resistance. *Weed Technology*, **15**, 257-263.

Bradford M M (1976). A rapid and sensitive method for the quantification of microgram quantities of protein utilising the principle of protein-dye binding. *Analytical Biochemistry* **72**, 248-254.

Brenchley W E (1913). The weeds of arable land in relation to the soil on which they grow. *Annals of Botany*, **27**, 141-166.

Brown A H D and Burdon J J (1987). Mating systems and colonizing success in plants. **In: Gray A J, Crawley M J and Edwards P J,** *eds. Colonization, succession and stability.* Oxford: Blackwell Scientific Publications. p. 115-131.

Burnet M W M, Loveys B R, Holtum J A M and Powles S B (1993). A mechanism of chlorotoluron resistance in *Lolium rigidum. Planta*, **190**, 182-189.

Buttke T M and Sandstrom P A (1994). Oxidative stress as a mediator of apoptosis. *Immunology Today*, 15, 7-10.

Carr J E, Davies L G, Cobb A H and Pallett K E (1986). Uptake, translocation and metabolism of fluazifop-butyl in *Setaria viridis. Annals of Applied Biology,* **114,** 339-348.

Cavan G and Moss S R (1997). Herbicide resistance and gene flow in black-grass (*Alopecurus myosuroides*) and wild oats (*Avena* spp.). *Proceedings of the 1997 Brighton Crop Protection Conference, Weeds,* **1**, 305-310.

Cavan G, Bliss P and Moss S R (1998). Localized origins of herbicide resistance in *Alopecurus myosuroides. Weed Research*, **38**, 239-245.

Chancellor R J and Froud-Williams R J (1986). Weed problems of the next decade in Britain. *Crop Protection*, **5**, 66-72.

Chapple C (1998). Molecular-genetic analysis of plant cytochrome P450-dependent monoxoygenases. *Annual Review of Plant Physiology and Plant Molecular Biology*, **49**, 311 – 343.

Chauvel B (1991). Polymorphisme génétique et sélection de la resistance aux urées substitutuées chez *Alopecurus myosuroides* Huds. PhD thesis. Université de Paris-sud, Centre D'Orsay.

Chauvel B and Gasquez J (1991). Study of the growth of herbicide resistant black-grass populations. **In: Caseley, J C, Cussan G W and Atkin R,** *eds*, *Herbicide resistance in weeds and crops*. Oxford: Butterworth-Heinemann. pp. 416-417.

Chauvel B and Gasquez J (1994). Relationships between genetic polymorphism and herbicide resistance within *Alopecurus myosuroides* Huds. *Heredity*, **72**, 336-344.

Chauvel B, Gasquez J, Doucey M A and Perreau F (1992). Selection for fenoxaprop-P-ethyl resistance within black-grass (*Alopecurus myosuroides* Huds.) populations. *Ixeme Colloque International sur la Biologie des Mauvaises Herbes, Dijon, ANPP Annales, 1992,* 487-496.

Chauvel B, Munier-Jolain N, Letouzé A and Grandgirard D (2000). Developmental patterns of leaves and tillers in a black-grass population (*Alopecurus myosuroides* Huds.). *Agronomie*, **20**, 247-257.

Clarke E D, Greenhow D T and Adams D (1998). Metabolism-related assays and their application to agrochemical research: reactivity of pesticides with glutathione and glutathione transferases. *Pesticide Science* **54**, 385-393.

Clarke J H (1987). Evaluation of herbicides for the control of *Alopecurus myosuroides* (black-grass) in winter cereals. Summary of results of ADAS trials 1985 and 1986 harvest years. *Proceedings of the British Crop Protection Conference, 1987, Weeds*, **2**, p. 375-382.

Clarke J H and Moss S R (1989). The distribution and control of herbicide resistant *Alopecurus myosuroides* (black-grass) in central and eastern England. *Proceedings of the Brighton Crop Protection Conference, 1989, Weeds,* **1**, 301-308.

Clarke J H and Moss S R (1991). The occurrence of herbicide resistant *Alopecurus myosuroides* (Black-grass) in the United Kingdom and strategies for its control. *Proceedings of the Brighton Crop Protection Conference, 1991, Weeds*, **3**, 1041-1048.

Clarke J H, Blair A M and Moss S R (1994). The testing and classification of herbicide resistant *Alopecurus myosuroides* (black-grass). *Aspects of Applied Biology: Sampling to make decisions*, **37**, 181-188.

Clewer A G and Scarisbrick D H (2001). *Practical statistics and experimental design for plant and crop science.* Chichester: John Wiley and Sons.

Cobb A H (1992). *Herbicides and plant physiology.* London: Chapman and Hall.

Cobb A H and Kirkwood R C (2000). *Herbicides and their mechanisms of action.* Sheffield: Sheffield Academic Press.

Cocker K M, Moss S R and Coleman J O D (1999). Multiple mechanisms of resistance to fenoxaprop-P-ethyl in United Kingdom and other European populations of herbicide-resistant *Alopecurus myosuroides* (black-grass). *Pesticide Biochemistry and Physiology*, **65**, 169-180.

Cocker K M, Coleman J O D, Blair A M, Clarke J H and Moss S R (2000). Biochemical mechanisms of cross-resistance to aryloxyphenoxypropionate and cyclohexanedione herbicides in populations of *Avena* spp. *Weed Research*, **40**, 323-334.

Cocker K M, Northcroft D S, Coleman J O D and Moss S R (2001). Resistance to ACCase-inhibiting herbicides and isoproturon in UK populations of *Lolium multiflorum*: mechanisms of resistance and implications for control. *Pest Management Science*, **57**, 587-597.

Cole D J (1994). Detoxification and activation of herbicides by plants. *Pesticide Science*, **42**, 209-222.

Cole D J and Edwards R (2000). Secondary metabolism of agrochemicals in plants. **In: Roberts T**, *ed. Metabolism of agrochemicals in plants*. Chichester: John Wiley and Sons Ltd. p. 107-154.

Cole D J, Cummins I C, Hatton P J, Dixon D and Edwards R (1997). Glutathione transferases in crops and major weeds. **In: Hatzios K K**, *ed. Regulation of enzymatic systems detoxifying xenobiotics in plants*. Dordrecht, The Netherlands: Kluwer Academic Press. p. 139-154.

Coleman J O D, Blake-Kalff, M M A and Emyr Davies, T G (1997). Detoxification of xenobiotics of plants: chemical modification and vacuolar compartmentation. *Trends in Plant Science*, **2**, 144-151.

Coupland D (1991). The role of compartmentation of herbicides and their metabolites in resistance mechanisms. **In: Caseley J C, Cussans G W and Atkin R K**, *eds. Herbicide resistance in weeds and crops*. Oxford: Butterworth-Heinemann. p. 263-278.

Cummins I, Moss S, Cole D J and Edwards R (1997a). Glutathione transferases in herbicide-resistant and herbicide-susceptible black-grass (*Alopecurus myosuroides*). *Pesticide Science*, **51**, 244-250.

Cummins I, Cole D J and Edwards R (1997b). Purification of multiple glutathione transferases involved in herbicide detoxification from wheat (*Triticum aestivum* L.) treated with the safener fenchlorazole-ethyl. *Pesticide Biochemistry and Physiology*, **59**, 35-49.

Cummins I, Cole D J and Edwards R (1999). A role for glutathione transferases functioning as glutathione peroxidases in resistance to multiple herbicides in black-grass. *Plant Journal*, **18**, 285-292.

Cyanamid (1997). *Crop Protection Product Guide 1997/8 – Stomp.* Hampshire: Cyanamid Agriculture UK.

Davies W J and Blackman P G (1989). Growth and development of plants in controlled environments and in the field. *Aspects of Applied Biology – Comparing Laboratory and Field Pesticide Performance*, **21**, 1-12.

Deege R, Förster H, Schmidt R R, Thielert W, Tice M A, Aagesen G J, Bloomberg J R and Santel H J (1995). BAY FOE 5043: A new low rate herbicide for preemergence grass control in corn, cereals, soybeans and other selected crops. *Proceedings of the Brighton Crop Protection Conference, 1995, Weeds,* **1**, 43-48.

De Kok L J, Maas F M, Godeke J, Haaksma A B and Kuiper P J C (1986). Glutathione, a tripeptide which may function as a temporary storage of excessive reduced sulphur in H_2S fumigated spinach plants. *Plant and Soil*, **91**, 349-352.

Dekker J (1999). ACCase inhibitors: Aryloxyphenoxy Carboxylic Acid sub-family. Online. Internet 1999.
Available: www.agron.iastate.edu/~weeds/Ag319-99/manage/herbicides/fops.html.

De Prado R, Menendez J, Tena M, Caseley J and Taberner, A (1991). Response to substituted ureas, triazines and chloroacetanilides in a biotype of *Alopecurus myosuroides* resistant to chlortoluron. *Proceedings of the Brighton Crop Protection Conference, 1991, Weeds,* **3**, 1065-1070.

Devine M D (1988). Environmental influences on herbicide performance: A critical evaluation of experimental techniques. *Proceedings of the EWRS Symposium Factors affecting herbicidal activity and selectivity, 1988.* pp. 219-226.

Devine M D and Eberlein C V (1997). Physiological, biochemical and molecular aspects of herbicide resistance based on altered target sites. **In: Roe R M, Burton J D and Kuhr R J,** *eds, Herbicide activity, toxicology, biochemistry and molecular biology.* Amsterdam: IOS Press. p. 159-186.

Devine M D and Preston C (2000). The molecular basis of herbicide resistance. **In: Cobb A H and Kirkwood R C,** *eds. Herbicides and their mechanisms of action.* Sheffield: Sheffield Academic Press. p. 72-104.

Devine M D, Duke S O and Fedtke C (1993). *Physiology of Herbicide Action.* New Jersey : PTR Prentice-Hall Inc.

Diggle A J and Neve P (2001). The population dynamics and genetics of herbicide resistance – a modelling approach. **In: Powles S B and Shaner D L,** *eds. Herbicide resistance and world grains.* Boca Raton, Florida: CRC Press. p. 61-99.

Dixon D P, Cummins I, Cole D J and Edwards R (1998). Glutathione-mediated detoxification systems in plants. *Current Opinion in Plant Biology*, **1**, 258-266.

Dupont (1998). *Lexus 50 DF Herbicide – 1998/9 Recommendations.* Stevenage: DuPont UK Limited.

Durst F and O'Keefe D P (1995). Plant cytochromes P450: an overview. *Drug Metabolism and Drug Interactions*, **12**, 171 – 187.

Edwards R (1995). Glutathione transferases in plants involved in herbicide detoxification. *Proceedings of the Brighton Crop Protection Conference, 1995, Weeds*, **3**, 823-832.

Edwards R (1997). Glutathione transferases and herbicide metabolism and selectivity. **In: De Prado R, Jorrin J and Garcia-Torres,** *eds. Weed and Crop Resistance to Herbicides.* Dordrecht, The Netherlands: Kluwer Academic Press. p.109-115.

Edwards R (2000). Towards understanding the function of glutathione transferases in plant metabolism. **In: Brunold *et al*** *eds. Sulfur nutrition and sulfur assimilation in higher plants.* Switzerland: Paul Haupt. 369-371.

Edwards R and Dixon D P (2000). The role of glutathione transferases in herbicide metabolism. **In: Cobb A H and Kirkwood R C,** *eds. Herbicides and their mechanisms of action.* Sheffield: Sheffield Academic Press. p. 39-71.

Edwards R, Dixon D P and Walbot V (2000). Plant glutathione *S*-transferases; enzymes with multiple functions in sickness and health. *Trends in Plant Science*, **5**, 193-198.

Eshdat Y, Holland D, Faltin Z, Ben-Hayim G (1997). Plant glutathione peroxidases. *Physiologia Plantarum,* **100**, 234-240.

Flury T, Adam D and Kreuz K (1995). A 2,4-D-inducible glutathione *S*-transferase from soybean (*Glycine max*). Purification, characterisation and induction. *Physiologia Plantarum*, **94**, 312 – 318.

Foyer C H (1993). Ascorbic acid. **In: Alscher R G and Hess J L** *eds. Antioxidants in higher plants*. Boca Raton: CRC Press. pp. 31-58.

Foyer C H and Halliwell B (1976). The presence of glutathione and glutathione reductase in chloroplasts: a proposed role in ascorbate metabolism. *Planta*, **133**, 21-25.

Foyer C H and Mullineaux P M (1994). *Causes of photooxidative stress and amelioration of defence systems in plants*. Boca Raton, Florida: CRC Press.

Foyer C H and Rennenberg H (2000). Regulation of glutathione synthesis and its role in abiotic and biotic stress defence. **In: Brunold C** ***et al****, eds. Sulfur Nutrition and Sulfur Assimilation in Higher Plants*. Bern, Switzerland: Paul Haupt. pp. 127-153.

Foyer C H and Fletcher J M (2001). Plant antioxidants: Colour me healthy. *Biologist*, **48**, 115-120.

Foyer C H and Noctor G (2001). The molecular biology and metabolism of glutathione. **In: Grill D** ***et al****, eds. Significance of Glutathione in Plant Adaptation to the Environment*. Dordrecht, The Netherlands: Kluwer Academic Publishers. pp. 27-56.

Foyer C H, Lelandais M and Kunert K J (1994a). Photooxidative stress in plants. *Physiologia Plantarum*, **92**, 616-717.

Foyer C H, Descourvières P and Kunert K J (1994b). Protection against oxygen radicals: an important defense mechanism studied in transgenic plants. *Plant, Cell and Environment*, **17**, 507-523.

Foyer C H, Theodoulou F L and Delrot S (2001). The functions of inter- and intracellular glutathione transport systems in plants. *Trends in Plant Science*, **6**, 486-492.

Frear D S (1995). Wheat microsomal cytochrome P450 monooxygenases: characterization and importance in the metabolic detoxification and selectivity of wheat herbicides. *Drug Metabolism and Drug Interactions*, **12**, 329-357.

Frear D S and Swanson H R (1970). Biosynthesis of *S*-(4-ethylamino-6-isopropylamino-2-s-triazino) glutathione. Partial purification and properties of glutathione *S*-transferase from corn. *Phytochemistry*, **9**, 2123-2132.

Frear D S, Swanson H R and Tanaka F S (1969). *N*-demethylation of substituted 3-(phenyl)-1-methylureas: isolation and characterization of a microsomal mixed function oxidase from cotton. *Phytochemistry*, **8**, 2157-2169.

Froud-Williams R J (1985). Dormancy and germination of arable grass-weeds. *Aspects of Applied Biology – The biology and control of weeds in cereals*, **9**, 9-18.

Gair R, Jenkins J E Emand Lester E (1991). *Cereal pests and diseases.* Ipswich: Farming Press. p. 79-152.

Garrod J F (1989). Comparative responses of laboratory and field grown test plants to herbicides. *Aspects of Applied Biology - Comparing Laboratory and Field Pesticide Performance*. **21**, 51-64.

Gasquez J and Darmency H (1991). Variability in herbicide response within weed species. *Proceedings of the Brighton Crop Protection Conference, 1991, Weeds*, **3**, 1023-1030.

Gajbhiye V T and Gupta S (2001). Adsorption-desorption behaviour of flufenacet in five different soils of India. *Pest Management Science*, **57**, 633-639.

Gill G S (1990). Evaluation of the Petri dish assay for screening diclofop-methyl resistance in partially resistant populations of annual ryegrass, *Lolium rigidum*. *Proceedings of the 9th Australian Weed Conference*, 220-223.

Gillham D J and Dodge A D (1987). Chloroplast superoxide and hydrogen peroxide scavenging systems from pea leaves: seasonal variations. *Plant Science* **50**, 105-109.

Glasgow L and Reynolds J (2000). Future weed management. *An abstract of the 3rd International Weed Science Congress, Foz Do Iguassh, Brazil, 6-11 June, 2000.* p. 97.

Graham J C (1993). *Introduction to A review of graminicide resistance – Monograph Number 1 by A Mortimer.* Belgium: Herbicide Resistance Action Committee.

Gray J A, Stoltenberg D E and Balke N E (1995). Absence of herbicide cross-resistance in two atrazine-resistant velvetleaf (*Abutilon theophrasti*) biotypes. *Weed Science*, **43**, 352-357.

Gray J A, Balke N E and Stoltenberg D E (1996). Increased glutathione conjugation of atrazine confers resistance in a Wisconsin Velvetleaf (*Abutilon theophrasti*) biotype. *Pesticide Biochemistry and Physiology*, **55**, 157-171.

Gressel J (1991). Why get resistance? It can be prevented or delayed. **In: Caseley J C, Cussans G W and Atkin R K**, *eds. Herbicide resistance in weeds and crops.* Oxford: Butterworth-Heinemann. p. 1-25.

Gressel J and Galun E (1994). Genetic controls of photooxidant tolerance. **In: Foyer C H and Mullineaux P M** *eds. Cause of Photooxidative stress and amelioration of defense systems in plants.* Boca Raton: CRC Press. pp. 237-273.

Gressel J and Segel L A (1978). The paucity of genetic adaptive resistance of plants to herbicides: Possible biological reasons and implications. *Journal of Theoretical Biology*, **75**, 349-371.

Gressel J and Segel L A (1990). Modelling the effectiveness of herbicide rotations and mixtures as strategies to delay or preclude resistance. *Weed Technology*, **4**, 186-198.

Griffith O W (1980). Determination of glutathione disulfide using glutathione reductase and 2-vinylpyridine. *Analytical Biochemistry*, **106**, 207-212.

Gronwald J W, Fuerst E P, Eberlein C V and Egli M A (1987). Effect of herbicide antidotes on glutathione content and glutathione *S*-transferase activity of sorghum roots. *Pesticide Biochemistry and Physiology*, **29**, 66-76.

Gronwald J W (1994). Resistance to photosystem II inhibiting herbicides. **In: Powles S B and Holtum J A M,** *eds, Herbicide resistance in plants: Biology and Biochemistry.* Boca Raton, Florida: Lewis/CRC Press. p. 27-60.

Gronwald J W and Plaisance K L (1998). Isolation and characterization of glutathione *S*-transferase isozymes from sorghum. *Plant Physiology*, **117**, 877-892.

Gronwald J W, Andersen R N and Yee C (1989). Atrazine resistance in Velvetleaf (*Abutilon theophrasti*) due to enhanced atrazine detoxification. *Pesticide Biochemistry and Physiology*, **34**, 149-163.

Gwynne D C and Murray R B (1985). *Weed biology and control in agriculture and horticulture.* London: Batsford Academic and Educational.

Hall A E (2001). *Crop Responses to Environment.* Boca Raton, Florida: CRC Press.

Hall, L M, Moss S R and Powles S B (1993). "Towards an understanding of resistance to APP herbicides in *Alopecurus myosuroides,*" Weed Management – towards tomorrow. *Proceedings of the 10th Council of Australian Weeds Science Societies and 14th Asian Pacific Weeds Science Society, Brisbane, Queensland, 1993,* 299-301.

Hall L M, Tardif, F J and Powles S B (1994a). Mechanisms of cross and multiple herbicide resistance in *Alopecurus myosuroides* and *Lolium rigidum. Herbicide resistance workshop, Edmonton, Alberta, Canada, 9-10th December 1993. Phytoprotection,* **75 (Suppl.)**, 17-23.

Hall L M, Holtum J A M and Powles, S B (1994b). Mechanisms responsible for cross resistance and multiple resistance. **In: Powles S B and Holtum J A M,** *eds. Herbicide resistance in plants: Biology and biochemistry.* Boca Raton, Florida: Lewis/CRC Press. p. 243-261.

Hall, L M, Moss S R and Powles S B (1995). Mechanism of resistance to chlorotoluron in two biotypes of the grass weed *Alopecurus myosuroides. Pesticide Biochemistry and Physiology*, **53**, 180-192.

Hall, L M, Moss S R and Powles S B (1997). Mechanisms of resistance to aryloxyphenoxypropionate herbicides in two resistant biotypes of *Alopecurus myosuroides* (blackgrass): Herbicide metabolism as a cross-resistance mechanism. *Pesticide Biochemistry and Physiology,* **57**, 87-98.

Halliwell B (1982). The toxic effects of oxygen on plants tissues – Volume 1. **In: Oberley L W,** *ed. Superoxide Dismutase.* Boca Raton, Florida: CRC Press.

Halliwell B and Foyer C H (1978). Properties and physical function of a glutathione reductase purified from spinach leaves by affinity chromatography. *Planta*, **139**, 9-17.

Halliwell B and Gutteridge J M C (1989). *Free radicals in biology and medicine.* Oxford: Clarendon.

Harper J L (1956). The evolution of weeds in relation to resistance to herbicides. *Proceedings of The Third British Weed Control Conference, Weeds, 1956,* **1**, 179-187.

Hathway D E (1989). *Molecular mechanisms of herbicide selectivity.* Oxford: Oxford University Press.

Hatton P J, Cole D J and Edwards R (1996). Influence of plant age on glutathione levels and glutathione transferases involved in herbicide detoxification in corn (*Zea mays* L.) and giant foxtail (*Setaria faberi* Herrm). *Pesticide Biochemistry and Physiology*, **54**, 199-209.

Hatton P J, Cummins I, Price L J, Cole D J and Edwards R (1998). Glutathione transferases and herbicide detoxification in suspension-cultured cells of giant foxtail *Setaria faberi* (giant foxtail). *Pesticide Science*, 53, 209-216.

Hausladen A and Alscher R G (1993). Glutathione. **In: Alscher R G and Hess J L** *eds. Antioxidants in higher plants.* Boca Raton: CRC Press. pp. 1-30.

Heap I M (1994). Identification and documentation of herbicide resistance. *Phytoprotection*, **75 (Suppl.)**, 85-90.

Heap I (1997). The occurrence of herbicide-resistant weeds worldwide. *Pesticide Science* **51**, 235-243.

Heap I (1999). International survey of herbicide-resistant weeds: lessons and limitations. *Proceedings of The 1999 Brighton Conference, Weeds,* **3**, 769-776.

Heap I (2001). The international survey of herbicide resistant weeds. Online. Internet. August 29, 2001. Available www.weedscience.com

Heap I (2002). The international survey of herbicide resistant weeds. Online. Internet. January 16, 2002. Available www.weedscience.com

Heap I and Knight R (1986). The occurrence of herbicide cross-resistance in a population of annual ryegrass, *Lolium rigidum*, resistant to diclofop-methyl. *Australian Journal of Agricultural Research*, **37**, 149-156.

Heap I and LeBaron H (2001). Introduction and overview of resistance. **In: Powles S B and Shaner D L,** *eds. Herbicide resistance and world grains.* Boca Raton, Florida: CRC Press. p. 1-22.

Hell R (1997). Molecular physiology of plant sulfur metabolism. *Planta*, **202**, 138-148.

Hendry G (1986). Why do plants have cytochrome P-450? Detoxification versus defence. *New Phytologist*, **102**, 239-247.

Herbicide Resistance Action Committee (undated). *Guideline to the management of herbicide resistance.* Switzerland: Herbicide Resistance Action Committee.

Herbiseed Technical Department, (1999). Personal Communication, Herbiseed Ltd UK.

Holm L G, Plucknett D L, Pancho J V and Herberger J P (1977). *The world's worst weeds.* Honolulu, Hawaii: The University Press of Hawaii.

Holm L G, Plucknett D L, Pancho J V and Herberger J P (1991). *The world's worst weeds.* Florida: Krieger Publishing Company.

Holm L, Doll J, Holm E, Pancho J and Herberger J (1997). *World weeds: Natural histories and distribution.* New York: John Wiley and Sons Inc.

Holt J S, Powles S B and Holtum J A M (1993). Mechanisms and agronomic aspects of herbicide resistance. *Annual Review of Plant Physiology and Plant Molecular Biology*, **44**, 203-229.

Holton T A and Cornish E C (1995). Genetics and biochemistry of anthocyanin biosynthesis. *Plant Cell*, **7**, 1071 – 1083.

Hossain M A, Nakano Y and Asada K (1984). Monodehydroascorbate reductase in spinach chloroplasts and its participation in regeneration of ascorbate for scavenging hydrogen peroxide. *Plant Cell Physiology*, **25**, 385-395.

Houghton J T, Meira Filho L G, Callander B A, Harris N, Kattenberg A and Maskell K (1996). *Climate change 1995: the science of climate change.* Cambridge: Cambridge University Press.

Hyde R J, Cheriton A K, Hallahan D L and Bowyer J R (1993). Herbicide resistance and cytochrome P-450. *Proceedings of the Brighton Crop Protection Conference, 1993, Weeds*, **2**, 549-553.

Hyde R J, Hallahan D L and Bowyer J R (1996). Chlorotoluron metabolism in leaves of resistant and susceptible biotypes of the grass weed *Alopecurus myosuroides*. *Pesticide Science*, **47**, 185-190.

Inzé D and Van Montagu M (1995). Oxidative stress in plants. *Current Opinion in Biotechnology*, **6**, 153-158.

Jablonkai I and Hatzios K K (1991). Role of glutathione and glutathione *S*-transferase in the selectivity of acetochlor in maize and wheat. *Pesticide Biochemistry and Physiology*, **41**, 221-231.

Jagtap V and Bhargava S (1995). Variation in the antioxidant metabolism of drought tolerant and drought susceptible varieties of *Sorghum bicolor* (L.) Moench. exposed to high light, low water and high temperature stress. *Journal of Plant Physiology*, **145**, 191-197.

Jasieniuk M, Brûlé-Babel A L and Morrison A N (1996). The evolution and genetics of herbicide resistance in weeds. *Weed Science*, **44** (1), 176-193.

Jiménez A, Hernández J A, del Río L A and Sevilla F (1997). Evidence for the presence of the ascorbate – glutathione cycle in mitochondria and peroxisomes of pea leaves. *Plant Physiology*, **114**, 275-284.

Jones O T G (1991). Cytochrome P450 and herbicide resistance. **In: Caseley J C, Cussans G W and Atkin R K,** *eds. Herbicide resistance in weeds and crops.* Oxford: Butterworth-Heinemann. p. 213-226.

Jutsum A R and Shaner D (1992). Herbicide resistance: The stance of the agrochemical industry. *Proceedings of the First International Weed Control Congress*, **2**, 244-246.

Kemp M S and Caseley J C (1987). Synergistic effect of 1-aminobenzotriazole on the phytotoxicity of chlortoluron and isoproturon in a resistant population of black-grass (*Alopecurus myosuroides*). *Proceedings of the Brighton Crop Protection Conference, 1987, Weeds*, **3**, 895-898.

Kemp M S, Moss S R and Thomas T H (1990). Herbicide resistance in *Alopecurus myosuroides*. **In: Green M B, LeBaron H and Moberg W,** *eds. Managing resistance to agrochemicals. From fundamentals to practical strategies.* Washington D.C.: American Chemical Society. p. 376-393.

Koch W (1968). Environmental factors affecting the germination of some annual grasses. *Proceedings of The Ninth British Weed Control Conference, 1968*, **1**, 14-19.

Koeppe M K, Barefoot A C, Cotterman C D, Zimmerman W T and Leep D C (1998). Basis of sensitivity of the herbicide flupyrsulfuron-methyl in wheat. *Pesticide Biochemistry and Physiology*, **59**, 105-117.

Knight H and Knight M R (2001). Abiotic stress signalling pathways: specificity and cross-talk. *Trends in Plant Science*, **6**, 262-267.

Knörzer O C and Böger P (1999). Antagonizing Peroxidizing Herbicides. **In: Böger P and Wakabayashi K** *eds. Peroxidizing Herbicides*. Berlin Heidelberg: Springer-Verlag. pp. 303-328.

Krähmer H and Russell P E (1994). General problems in glasshouse to field transfer of pesticide performance. **In: Hewitt H G, Caseley J, Copping L G, Grayson B T and Tyson D** *eds. Comparing glasshouse and field pesticide performance II – Monograph No 59.* Farnham: British Crop Protection Council. pp.3-16.

Kraus T E and Fletcher R A (1994). Paclobutrazol protects wheat seedlings from heat and paraquat injury. Is detoxification of active oxygen involved? *Plant Cell Physiology*, **35**, 45-52.

Kröniger W, Rennenberg H and Polle A (1993). Developmental changes of CuZn- and Mn-superoxide dismutase isozymes in seedlings and needles of Norway spruce (*Picea abies* L.). *Plant and Cell Physiology* **34**, 1145-1149.

Kunert K J and Foyer C (1993). Thiol/disulfide exchange in plants. **In: De Kok L J** *ed. Sulfur nutrition and assimilation in higher plants.* The Hague: SPB Academic. pp. 139-151.

Lamoureux G L and Rusness D G (1986). Xenobiotic conjugation in higher plants. **In: Paulson G D, Caldwell J, Huston D H and Menn J J**, *eds. Xenobiotic conjugation chemistry.* Washington D.C.: American Chemical Society. p. 62-105.

Lamoureux G L and Rusness D G (1989). The role of glutathione and glutathione *s*-transferases in pesticide metabolism, selectivity and mode of action in plants and insects. **In: Dolphin D, Poulson R and Avramovic O** *eds. Glutathione: Chemical, Biochemical and Medical aspects, Part B.* New York: John Wiley & Sons. 153-196.

Lamoureux G L and Rusness D G (1993). Glutathione in the metabolism and detoxification of xenobiotics in plants. **In: Dekok L J, Stulen H *et al eds.*** *Sulphur nutrition and assimilation in higher plants.* The Hague: SPB Academic Publishers. 221-237.

Lamoureux G L, Shimabukuro R H and Frear D S (1991). Glutathione and glucoside conjugation in herbicide selectivity. **In: Caseley J C, Cussans G W and Atkin R K,** *eds. Herbicide resistance in weeds and crops.* Oxford: Butterworth-Heinemann. p. 227-261.

Langer R H M (1979). *How grasses grow – Studies in Biology No 34.* 2nd Edition. London: Edward Arnold.

Langer R H M and Hill G D (1991). *Agricultural Plants.* 2nd Ed. Cambridge: Cambridge University Press. p. 40-139.

Leavitt J R C and Penner D (1979). In-vitro conjugation of glutathione and other thiols with acetanilide herbicides and EPTC sulfoxide and the action of the herbicide antidote R-25788. *Journal of Agricultural Food Chemistry*, **27**, 533-536.

LeBaron H M and Gressel J (1982). *Herbicide resistance in plants.* New York: John Wiley.

LeBaron H and McFarland J (1990). Herbicide resistance in weeds and crops: and overview and prognosis. **In: Green M B, LeBaron H M and Moberg W K,** *eds. Managing resistance to agrochemicals: from Fundamental research to practical strategies.* Washington D.C.: American Chemical Society. p. 336-352.

Letouzé A and Gasquez J (1999). A rapid reliable test for screening aryloxyphenoxypropionic acid resistance within *Alopecurus myosuroides* and *Lolium* spp. populations. *Weed Research*, **39**, 37-48.

Letouzé A and Gasquez J (2000). A pollen test to detect ACCase target-site resistance within *Alopecurus myosuroides* populations. *Weed Research*, **40**, 151-162.

Letouzé A, Gasquez J, Vaccara D, Orlando D, Leterrier J L, Roy C and Bouvard-Derieux E (1997). Development of new reliable quick tests and state of grass-weed herbicide resistance in France. *Proceedings of the 1997 Brighton Crop Protection Conference, Weeds*, **1**, 325-330.

Lee G J and Vierling E (2000). A small heat shock protein cooperates with heat shock protein 70 systems to reactivate a heat-denatured protein. *Plant Physiology*, **122**, 189 – 197.

Luwe M W F, Takahama U and Heber U (1993). Role of ascorbate in detoxifying ozone in the apoplast of spinach (*Spinacia oleracea* L.) leaves. *Plant Physiology*, **101**, 969-976.

Macnicol P K and Randall R J (1987). Changes in the levels of major sulfur metabolites and free amino-acids in pea cotyledons recovering from sulfur deficiency. *Plant Physiology*, **83**, 354-359.

Mahan J R, McMichael B L and Wanjura D F (1995). Methods for reducing the adverse effects of temperature stress on plants: A review. *Environmental and Experimental Botany*, **35**, 251-258.

Marrs K (1996). The functions and regulation of glutathione *S*-transferases in plants. *Annual Review in Plant Physiology*, **47**, 127-158.

Martinoia E, Grill E, Tommasini R, Kreuz K and Amrhein N (1993). ATP-dependent glutathione-*S*-conjugate 'export' pump in the vacuolar membrane of plants. *Nature*, **346**, 247-249.

Mauch F and Dudler R (1993). Differential induction of distinct glutathione *S*-transferases of wheat by xenobiotics and by pathogen attack. *Plant Physiology*, **102**, 1193-1201.

Maxwell B D and Mortimer A M (1994). Selection for herbicide resistance. **In: Powles S B and Holtum J A M,** *eds. Herbicide resistance in plants: Biology and biochemistry.* Boca Raton, Florida: Lewis/CRC Press. p. 1-25.

May M J, Vernoux T, Leaver C, Van Montagu M and Inzé D (1998). Glutathione homeostasis in plants: implications for environmental sensing and plant development. *Journal of Experimental Botany*, **49**, 649-667.

McFadden J J, Gronwald J W and Eberlein C V (1990). *In vitro* hydroxylation of bentazon by microsomes from naphthalic anhydride-treated corn shoots. *Biochemical and Biophysical Research Communications*, **168**, 206-213.

McKersie B D and Leshem Y Y (1994). *Stress and stress coping in cultivated plants*. Dordrect, The Netherlands: Kluwer Academic Publishers.

Menendez J and De Prado R (1996). Diclofop-methyl cross-resistance in a chlorotoluron-resistant biotype of *Alopecurus myosuroides*. *Pesticide Biochemistry and Physiology*, **56**, 123-133.

Menendez J, De Prado R, Jorrin R and Taberner A (1993). Penetration, translocation and metabolism of diclofop-methyl in chlorotoluron – resistant and –susceptible biotypes of *Alopecurus myosuroides*. *Proceedings of the Brighton Crop Protection Conference, 1993, Weeds*, **1**, 213-220.

Milner L J, Reade J P H and Cobb A H (1999). An investigation of glutathione *S*-transferase activity in *Alopecurus myosuroides* Huds. (black-grass) in the field. *Proceedings of the Brighton Conference, 1999, Weeds*, **1**, 173-178.

Milner L J, Reade J P H and Cobb A H (2001). Developmental changes in glutathione *S*-transferase activity in herbicide-resistant populations of *Alopecurus myosuroides* Huds. (black-grass) in the field. *Pest Management Science*, **57**, 1100-1106.

Mills C E and Ryan P J (1995). The control of herbicide-resistant *Alopecurus myosuroides* (black-grass). *Proceedings of the Brighton Crop Protection Conference, 1995, Weeds*, **3**, 1153-1160.

Ministry of Agricultre, Fisheries and Food (1994). *Fertiliser recommendations for agricultural and horticultural crops – RB209*. 6th Edition. London: HMSO.

Monsanto, Rhône-Poulenc, Ciba and Touche Ross Agriculture (1995). *Black-grass control – the total approach.* A booklet put together by members of Monsanto, Rhône-Poulenc, Ciba and Touche Ross Agriculture. May, Winfield Ltd.

Mortimer A M (1990). The biology of weeds. **In: Hance R J and Holly K** *eds. Weed Control Handbook: Principles.* Oxford: Blackwell Scientific Publications. p. 1-42.

Mortimer A M (1993). *A review of graminicide resistance – Monograph Number 1.* Belgium: Herbicide Resistance Action Committee.

Mortimer A M, Ulf-Hansen P F and Putwain P D (1991). Modelling herbicide resistance – a study of ecological fitness. **In: Denholm I, Devonshire A L and Hollomans D W,** *eds. Achievements and developments in combating pesticide resistance.* Amsterdam: Elsevier. p.148.

Moss S R (1979). The influence of tillage and method of straw disposal on the survival and growth of black-grass (*Alopecurus myosuroides*), and its control by chlorotoluron and isoproturon. *Annals of Applied Biology*, **91**, 91-100.

Moss S R (1980). Some effects of burning cereal straw on seed viability, seedling establishment and control of *Alopecurus myosuroides* Huds. *Weed Research*, **20**, 271-276.

Moss S R (1981). The agro-ecology and control of black-grass, *Alopecurus myosuroides* Huds., in modern cereal growing systems. *ADAS Quarterly Review 1975-80.* London: HMSO. p. 170-191.

Moss S R (1983). The production and shedding of *Alopecurus myosuroides* Huds. seeds in winter cereal crops. *Weed Research*, **23**, 45-51.

Moss S R (1985). The survival of *Alopecurus myosuroides* seeds in soil. *Weed Research*, **25**, 201-211.

Moss S R (1987a). Competition between black-grass (*Alopecurus myosuroides*) and winter wheat. *Proceedings of the British Crop Protection Conference, 1987, Weeds*, **2**, 367-374.

Moss S R (1987b). Herbicide resistance in black-grass (*Alopecurus myosuroides*). *Proceedings of the British Crop Protection Conference, 1987, Weeds*, **3**, 879-886.

Moss S R (1990a). The seed cycle of *Alopecurus myosuroides* in winter cereals: a quantitative analysis. *Proceedings of the EWRS Symposium 1990, Integrated Weed Management in Cereals*, 27-36.

Moss S R (1990b). Herbicide cross-resistance in slender foxtail (*Alopecurus myosuroides*). *Weed Science*, **38**, 492-496.

Moss S R (1992). Herbicide resistance in the weed *Alopecurus myosuroides* (black-grass): the current situation. **In: Denholm I, Devonshire A L and Holloman D W**, *eds. Achievements and developments in combating resistance.* London: Elsevier Applied Science Publishers. p. 28-40.

Moss S R (1995). Techniques for determining herbicide resistance. *Proceedings of the Brighton Crop Protection Conference, 1995, Weeds*, **2**, 547-556.

Moss S R (1996). *Comparative resistance tests on black-grass – protocol.* IACR Rothamsted.

Moss S R *ed.* (1997). *Revised guidelines for preventing and managing herbicide-resistant grass-weeds*. London: Home Grown Cereals Authority/Weed Resistance Action Group.

Moss S R (1999). Rothamsted Rapid Resistance Test: for detecting herbicide-resistance in black-grass, wild-oats and Italian rye-grass. Hertfordshire: IACR Rothamsted.

Moss S R and Clarke J H (1992). Herbicide resistance in black-grass (*Alopecurus myosuroides). HGCA Report No. 62.* London: Home Grown Cereals Authority.

Moss S R and Clarke (1995). *Inheritance of herbicide resistance in black-grass (Alopecurus myosuroides) and responses of the weed to a range of herbicides, HGCA Project Report No.116.* London: Home Grown Cereals Authority.

Moss S R, Clarke J H, Blair, A M, Culley T N, Read M A, Ryan P J and Turner M (1999). The occurrence of herbicide-resistant grass-weeds in the United Kingdom and a new system for designating resistance in screening assays. *Proceedings of the 1999 Brighton Conference, Weeds,* **1**, 179-184.

Moss S R and Cussans G W (1985). Variability in the susceptibility of *Alopecurus myosuroides* (black-grass) to chorotoluron and isoproturon. *Aspects of Applied Biology*, **9**, 91-98.

Moss S R and Cussans G W (1991). The development of herbicide-resistant populations of *Alopecurus myosuroides* (black-grass) in England. **In: Caseley, J C, Cussans G W and Atkin, R K,** *eds. Herbicide resistance in weeds and crops.* Oxford: Butterworth-Heinemann. p. 45-55.

Moss S R, Horswell J, Froud-Williams R J and Ndoping M M (1993). Implications of herbicide resistant *Lolium multiflorum* (Italian rye-grass). *Aspects of Applied Biology*, **35**, 53-60.

Moss S R and Rubin B (1993). Herbicide-resistant weeds: a worldwide perspective. *Journal of Agricultural Science* **120**, 141-148.

Mostowska A and Gwóźdź E A (1995). Reaction of photosynthetic apparatus to oxidative stress. *Post Bio Kom*, **22**, 43-63.

Murakami Y , Tsuyama M, Kobayashi Y, Kodama H and Iba K (2000). Trienoic fatty acids and plant tolerance of high temperature. *Science*, **287**, 476 – 479.

Murray B G, Friesen L F, Beauliea K J and Morrison I N. (1996). A seed bioassay to identify acetyl-CoA carboxylase inhibitor resistant wild oat (*Avena fatua*) populations. *Weed Technology*, **10**, 85-89.

Muzik T J (1976). Influence of environmental factors on toxicity to plants. **In: Audus L J,** *ed. Herbicides Physiology, Biochemistry, Ecology. 2nd Edition, Volume 2.* London: Academic Press. pp. 203-247.

Nakano Y and Asada K (1980). Spinach chloroplasts scavenge hydrogen peroxide on illumination. *Plant Cell Physiology*, **21**, 1295-1307.

Nakano Y and Asada (1981). Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. *Plant Cell Physiology*, **22**, 867-880.

Naylor R E L (1970). The prediction of black-grass infestations. *Weed Research*, **10**, 296-299.

Naylor R E L (1972a). Biological flora of the British Isles. *Journal of Ecology*, **60**, 611-622.

Naylor R E L (1972b). The nature and consequence of interference by *Alopecurus myosuroides* Huds. on the growth of winter wheat. *Weed Research*, **12**, 137-143.

Naylor R E L (1972c). Aspects of the population dynamics of the weed *Alopecurus myosuroides* Huds. in winter cereal crops. *Journal of Applied Ecology*, **9**, 127-139.

Niemann P and Pestemer W (1984). Resistenz verschiedener herkunfte von ackerfuchsschwanz (*Alopecurus myosuroides*) gegenuber herbizidbehandlungen. *Nachrichtenblatt des Deutschen Pflanzenschutzdienstes*, **36**, 113-118.

Noctor G and Foyer C H (1998). Ascorbate and glutathione. *Annual Review of Plant Physiology and Plant Molecular Biology*, **49**, 249-279.

Noctor G, Velijovic-Jovanovic S and Foyer C H (2000). Peroxide processing in photosynthesis: antioxidant coupling and redox signalling. *Philosophical Transactions of the Royal Society, London B*, **355**, 1-11.

Orson J H (1991). *Variability in the control of black-grass with herbicides in winter cereals – harvest years 1987-1989. HGCA Project Report No. 27.* London: Home Grown Cereals Authority.

Orson J H (1999). The cost to the farmer of herbicide resistance. *Weed Technology*, **13**, 607-611.

Orson J H and Harris D (1997). The technical and financial impact of herbicide resistant black-grass (*Alopecurus myosuroides*) on individual farm businesses in England. *Proceedings of the 1997 Brighton Crop Protection Conference, Weeds,* **3**, 1127-1132.

Owen W J (1987). Herbicide detoxification and selectivity. *Proceedings of the 1987 British Crop Protection Conference, Weeds,* **1**, 309-318.

Pastori G M and del Rio L A (1994). An activated-oxygen-mediated role for peroxisomes in the mechanism of senescence of *Pisum sativum* leaves. *Planta* **193**, 385-391.

Peter Tayler, Cyanamid, Personal Communication, (2000).

Polle A and Rennenberg H (1994). Photooxidative stress in tress. **In: Foyer C and Mullineaux P** *eds. Causes of Photooxidative stress and amelioration of defence systems in plants.* USA: CRC Press. pp. 1999-218.

Powles S B and Preston C (1995). *Herbicide cross resistance and multiple resistance in plants – Monograph Number 2.* Belgium: Herbicide Resistance Action Committee.

Powles S B, Preston C, Bryan I B and Jutsum A R (1997). Herbicide resistance: impact and management. **In: Sparks D L,** *ed. Advances in Agronomy* **58**, 57-93.

Preston C and Powles S B (1997). Light-dependent enhanced metabolism of chlorotoluron in a substituted urea herbicide-resistant biotypes of *Lolium rigidum* Gaud. *Planta,* **201**, 202-208.

Preston C, Tardif F J, Christopher J T and Powles S B (1996). Multiple resistance to dissimilar herbicide chemistries in a biotype of *Lolium rigidum* due to enhanced activity of several herbicide degrading enzymes. *Pesticide Biochemistry and Physiology,* **54**, 123-134.

Putwain P D (1982). Herbicide resistance in weeds – an inevitable consequence of herbicide use? *Proceedings of The British Crop Protection Conference, Weeds, 1982,* **2**, 719-728.

Queitsch C, Hong S, Vierling E and Lindquist S (2000). Heat shock protein 101 plays a crucial role in thermotolerance in arabidopsis. *Plant Cell*, **12**, 479 – 492.

Rabinowitch H D and Fridovich I (1983). Superoxide radicals, superoxide dismutases and oxygen toxicity in plants. *Photochemistry and Photobiology*, **37**, 679-690.

Read M A, Palmer J J and Howard S (1997). An integrated strategy for the successful management of herbicide resistant *Alopecurus myosuroides* (black-grass) in the UK. *Proceedings of the 1997 Brighton Crop Protection Conference, Weeds*, **1**, 343-350.

Reade J P H and Cobb A H (1999). Purification, characterization and comparison of glutathione *S*-transferases from black-grass (*Alopecurus myosuroides* Huds) biotypes. *Pesticide Science*, **55**, 993-999.

Reade J P H and Cobb A H (2002). New, quick tests for herbicide resistance in black-grass (*Alopecurus myosuroides* Huds.) based on increased glutathione *S*-transferase activity and abundance. *Pest Management Science*, **58**, 26-32.

Reade J P H, Hull M R and Cobb A H (1997). A role for glutathione *S*-transferases in herbicide resistance in black-grass (*Alopecurus myosuroides*). *Proceedings of the Brighton Crop Protection Conference, 1997, Weeds*, **2**, 777-782.

Reade J P H, Belfield J L and Cobb A H (1999). Rapid tests for herbicide resistance in blackgrass based on elevated glutathione *S*-transferase activity and abundance. *Proceedings of the 1999 Brighton Conference, Weeds*, **1**, 185-190.

Rennenberg H and Lamoureux G L (1990). Physiological processes that modulate the concentration of glutathione in plant cells. **In: Rennenberg *et al* eds.** *Sulfur nutrition and sulfur assimilation in higher plants.* The Hague: SPB Academic Publishing. pp. 53-67.

Rennenberg H and Brunold C (1994). Significance of glutathione in plants under stress. *Progress in Botany*, **55**, 142-156.

Rensburg L van and Kruger G H J (1994). Evaluation of components of oxidative stress metabolism for use in selection of drought tolerant cultivars of *Nicotiana tobacum* L. *Journal of Plant Physiology*, **101**, 7-12.

Richter J and Powles S B (1993). Pollen expression of herbicide target site resistance genes in annual ryegrass (*Lolium rigidum*). *Plant Physiology*, **102**, 1037-1041.

Roberts T R, Hutson D H, Lee P W, Nicholls P H and Plimmer J R (1998) *eds. Metabolic pathways of agrochemicals Part 1: Herbicides and plant growth regulators.* Cambridge: The Royal Society of Chemistry.

Roxas V P, Smith R K, Allen E R and Allen R D (1997). Overexpression of glutathione *S*-transferase/glutathione peroxidase enhances the growth of transgenic tobacco seedlings during stress. *Nature Biotechnology*, **15**, 988-991.

Rubin B (1991). Herbicide resistance in weeds and crops, progress and perspectives. **In: Caseley J C, Cussans G W and Atkin R K**, *eds. Herbicide resistance in weeds and crops*. Oxford: Butterworth-Heinemann. p. 387-414.

Rubin B (1992). Qualitative and quantitative methods for identifying and monitoring herbicide-resistant weeds. **In: Denholm I, Devonshire A L and Holloman D W**, *eds. Achievements and developments in combating resistance.* London: Elsevier Applied Science.

Ryan P J and Mills C E (1997). Quantification of the development of herbicide-resistant black-grass (*Alopecurus myosuroides*) under herbicide-based resistance management regimes. *Proceedings of the Brighton Crop Protection Conference-Weeds* **3**, 1141-1146.

Sabehat A, Weiss D and Lurie S (1998). Heat-shock proteins and cross-tolerance in plants. *Physiologia Plantarum*, **103**, 437 – 441.

Sairam R K, Deshmukh P S and Shukla D S (1997). Tolerance of drought and temperature stress in relation to increased antioxidant enzyme activity in wheat. *Journal of Agronomy and Crop Science*, **178**, 171-178.

Sairam R K, Srivastava G C and Saxena D C (2000). Increased antioxidant activity under elevated temperatures: a mechanism of heat stress tolerance in wheat genotypes. *Biologica Plantarum*, **43**, 245-251.

Sánchez-Fernández R, Fricker M, Corben L B, White N S, Sheard N, Leaver C J, Van Montegue M, Inzé D and May M J (1997). Cell proliferation and hair tip growth in the *Arabidopsis* root are under mechanistically different forms of redox control. *Proceedings of the National Academic Science USA, 1997,* **94**, 2745-2750.

Scandalios J G (1990). Response of plant antioxidant defense gene to environmental stress. **In: Scandalios J G,** *ed. Genomic responses to environmental stress*. San Diego: Academic Press. pp. 1-41.

Scandalios J G (1993). Oxygen stress and superoxide dismutases. *Plant Physiology*, **101**, 7-12.

Scarponi L, Perucci P and Marinetti L (1991). Conjugation of 2-chloroacetanilide herbicides with glutathione: Role of molecular structures and of glutathione *s*-transferase enzymes. *Journal of Agricultural Food Chemistry* **39**, 2010-2013.

Schmidt R R (1997). Herbicide Resistance Action Committee classification of herbicides according to mode of action. *Proceedings of the Brighton Crop Protection Conference, 1997, Weeds,* **3**, 1133-1140.

Schöffl F and Prändl R (1999). De-repression of heat shock protein synthesis in transgenic plants. **In: Smallwood M F, Calvert C M and Bowles D J,** *ed. Plant Responses to Environmental Stress*. Oxford: BIOS Scientific Publishers Ltd. p. 65 - 73

Schöner S and Krause G H (1990). Protective systems against active oxygen species in spinach: response to cold acclimation in excess light. *Planta*, **180**, 383-389.

Schuler M A (1996). Plant cytochrome P450 monooxygenases. *Critical Reviews of Plant Science*, **15**, 235 – 284.

Schwanz P and Polle A (1998). Antioxidative systems, pigment and protein contents in leaves of adult Mediterranean oak species (*Quercus pubescens* and *Q. ilex*) with lifetime exposure to elevated CO_2. *New Phytologist*, **140**, 411-423.

Scott N M (1981). Evaluation of sulphate status of soils by plant and soil tests. *Journal of Science, Food and Agriculture* **32**, 193-199.

Selinioti J E, Manetas Y and Gavalas N A (1986). Cooperative effects of light and temperature on the activity of phosphonenolpyruvate carboxylase from *Paniculatus* L. *Plant Physiology*, **82**, 518-522.

Shaner D L (1995). Herbicide resistance: Where are we? How did we get here? Where are we going? *Weed Technology*, **9**, 850-856.

Sharples K and Cobb A (1996). An investigation into chlorotoluron resistance in blackgrass (*Alopecurus myosuroides*). *Pesticide Science*, **46**, 284-286.

Sharples C R, Sanders G E and Cobb A H (1993). Growth analysis of chlorotoluron resistant and susceptible black-grass. *Proceedings of the Brighton Crop Protection Conference, 1993, Weeds*, **2**, 627-628.

Sharples C R, Hull M R and Cobb A H (1995). The effect of herbicide safeners on chlorotoluron resistant susceptible and resistant black-grass *(Alopecurus myosuroides* Huds.). *Proceedings of the Brighton Crop Protection Conferences-Weeds* **1**, 359-360.

Sharples C R, Hull M R and Cobb A H (1997). Growth and photosynthetic characteristics of two biotypes of the weed black-grass (*Alopecurus myosuroides* Huds.) resistant and susceptible to the herbicide chlorotoluron. *Annals of Botany*, **79**, 455-461.

Shimabukuro R H (1985). Detoxification of herbicides. **In: Duke S O**, *ed. Herbicide Physiology Volume II.* Boca Raton, Florida: CRC Press. p. 215-240.

Shimabukuro R H, Lamoureux G L and Frear D S (1978). Glutathione conjugation: A mechanism of herbicide detoxification and selectivity in plants. **In: Pallos F M and Casida J E**, *eds. Chemistry and Action of Herbicide Antidotes.* New York: Academic Press. p. 133-150.

Shimabukuro R H, Frear D S, Swanson H R and Walsh W C (1971). Glutathione conjugation. An enzymatic basis for atrazine resistance in corn. *Plant Physiology*, **47**, 10-14.

Siddall C J and Cousins S F B (1982). Annual and perennial grass weed control in sugar beet following sequential and tank-mix application of fluazifop-butyl and broad-leaf herbicides. *Proceedings of the British Crop Protection Conference, 1982, Weeds*, 2, 827-33.

Sies H (1991). *Ed. Oxidative Stress-Oxidants and Antioxidants.* New York: Academic Press.

Simon J P (1979). Adaptation and acclimation of higher plants at the enzyme level: latitudinal vanations of thermal properties of NAD malate dehydrogenase in *Lathyrus japonicus* Willd. (*Leguminosae*). *Oecologia*, **39**, 273-287.

Simon E W, Dormer K J and Hartshorne J N (1980). *Lowson's Botany.* 15th Edition. London: University Tutorial Press Ltd. pp. 303-306.

Simon J P, Potvin C and Blanchard M (1983). Thermal adaptation and acclimation of higher plants at the enzyme level: Kinetic properties of NAD malate dehydrogenase and glutamate oxaloacetate transaminase in two genotypes of *Arabidopis thaliana* (*Brassicaceae*). *Oecologia*, **60**, 143-148.

Sinclair W, Greenwood M, Marshall G, Moss S R, Walter H, Mortimer A M and Putwain P (1999). PCR and sequence based strategies for the detection of ACCase inhibitor resistance in grass weeds. *Proceedings of the 1999 Brighton Conference, Weeds*, **3**, 791-796.

Skipsey M, Andrews C J, Townson J K, Jepson I and Edwards R (1997). Substrate and thiol specificity of a stress-inducible glutathione transferase from soybean. *FEBS Letters*, **409**, 370 – 374.

Smallwood M F, Calvert C M and Bowles D J (1999). *Plant responses to environmental stress.* Oxford: BIOS Scientific Publishers Ltd.

Smeda R J, Barrentine W L, Snipes C E and Rippee J H (1997). Identification of Johnson grass resistance to graminicides and alternative control methods. **In: De Prado R, Jorrin R and Garcia-Torres L**, *eds. Weed and crop resistance to herbicides.* Dordrect, The Netherlands: Kluwer Academic Publishers. p. 262-263.

Smith I K (1975). Sulfate transport in cultured tobacco cells. *Plant Physiology* **55**, 303-307.

Smith I K (1985). Stimulation of glutathione synthesis in photorespiring plants by catalase inhibitors. *Plant Physiology*, **79**, 1044-1047.

Smith I K, Kendall A C, Keys A J, Turner J C and Lea P J (1984). Increased levels of glutathione in a catalase-deficient mutant of barley (*Hordeum vulgare*). *Plant Science Letters*, **37**, 29-33.

Snaydon R W (1982). Weeds and crop yield. *Proceedings of the 1982 Brighton Crop Protection Conference, Weeds,* **2**, 729-739.

Stauss R (1994). *Compendium of growth stage identification keys for mono- and dicotyledonous – extended BBCH scale.* Ciba-Geigy AG:Basel.

Takahashi Y, Hasezawa S, Kusaba M and Nagata T (1995). Expression of the auxin-regulated *parA* gene in transgenic tobacco and nuclear localization of its gene product. *Planta*, **196**, 111-117.

Tal A, Romano L, Stephenson G R, Schwan A L and Hall J C (1993). Glutathione conjugation: A detoxification pathway for fenoxaprop-ethyl in barley, crabgrass, oat and wheat. *Pesticide Biochemistry and Physiology*, **46**, 190-199.

Tal J A, Hall J C and Stephenson G R (1995). Non-enzymatic conjugation of fenoxaprop-ethyl with glutathione and cysteine in several grass species. *Weed Research*, **35**, 133-139.

Tal A, Kotoula-Syka E and Rubin B (2000). Seed-bioassay to detect grass weeds resistant to acetyl coenzyme A carboxylase inhibiting herbicides. *Crop Protection*, **19**, 467-472.

Tanaka K and Sugahara K (1980). Role of superoxide dismutase in defense against SO_2 toxicity and an increase in superoxide dismutase activity with SO_2 fumigation. *Plant Cell Physiology*, **21**, 601-611.

Tardif F J and Powles S B (1993). Target site-based resistance to herbicides inhibiting acetyl-CoA carboxylase. *Proceedings of the Brighton Crop Protection Conference, 1993, Weeds*, **2**, 533-539.

Tardif F J and Powles S B (1994). Herbicide multiple-resistance in a *Lolium rigidum* biotype is endowed by multiple mechanisms: Isolation of a subset with resistant acetyl-CoA carboxylase. *Physiologia Plantarum*, **91**, 488-494.

Tardif F J, Preston C and Powles S B (1996). Mechanisms of multiple herbicide resistance in *Lolium rigidum*. **In: De Prado R, Jorrin R and Garcia-Torres L**, *eds. Weed and crop resistance to herbicides*. Dordrect, The Netherlands: Kluwer Academic Publishers. P. 117-124.

Teaney S R, Armstrong L, Bentley K, Cotterman D, Leep D, Liang P H, Powley C, Summers J, Cranwell S, Lichtner R and Stichbury R (1995). DPX-KE459 – A new sulfonylurea for postemergence grass and broadleaf weed control in cereals. *Proceedings of the Brighton Crop Protection Conference, 1995, Weeds*, **1**, 49-56.

Thurston J M (1964). Germination of *Alopecurus myosuroides* Huds. (Black-grass). *Proceedings of The Seventh British Weed Control Conference, 1964*, **1**, 349-351.

Tomlin C D S *ed.* (2000) *The Pesticide Manual*. Farnham: The British Crop Protection Council.

Truelove B and Hensley J R (1982). Methods of testing for herbicide resistance. **In: LeBaron H M and Gressel J**, *eds*, *Herbicide resistance in plants*. New York: Wiley-Interscience. p. 117-131.

van Oorschot J L P (1991). Chloroplastic resistance of weeds to triazines in Europe. **In: Caseley J C, Cussans G W and Atkin R K**, *eds*. *Herbicide resistance in weeds and crops*. Oxford: Butterworth-Heinemann. p. 87-101.

van Oorschot J L P and van Leeuwen P H (1992). Use of fluorescence induction to diagnose resistance of *Alopecurus myosuroides* Huds. (black-grass) to chlorotoluron. *Weed Research*, **32**, 473-482.

Vanacker H, Carver T L and Foyer C H (2000). Early H_2O_2 accumulation in mesophyll cells leads to induction of glutathione during the hypersensitive response in the barley-powdery mildew interaction. *Plant Physiology*, **123**, 1289-1300.

Vierling E (1991). The roles of heat shock proteins in plants. *Annual Review of Plant Physiology and Plant Molecular Biology*, **42**, 579 – 620.

Wallgren B and Aamisepp A (1977). Biology and control of *Alopecurus myosuroides* Huds. and *Apera spica-venti* L.. *Proceedings of the European Weed Research Society Symposium: Methods of weed control and their integration*, 229-241.

Werck-Reichart D (1995). Herbicide metabolism and selectivity: Role of cytochrome P450. *Proceedings of the Brighton Crop Protection Conference, 1995, Weeds*, **3**, 813-822.

Whitehead R *ed.* (2001). *The UK Pesticide Guide 2001*. Oxon/Farnham: CAB International/British Crop Protection Council.

Willis A D, Mortimer A, Putwain P D and Moss S R (1997). Variation in cross resistance in black-grass (*Alopecurus myosuroides*) within and between populations: Implications for management. *Proceedings of the Brighton Crop Protection Conference, 1997, Weeds*, **2**, 675-676.

Winkler B S, Orselli S M and Rex T S (1994). The redox couple between glutathione and ascorbic acid: a chemical and physiological perspective. *Free Radical Biology and Medicine*, **17**, 333-349.

Wolf A E, Dietz K-J and Schroder P (1996). Degradation of glutathione *S*-conjugates by a carboxypeptidase in the plant vacuole. *FEBS Letters*, **384**, 31-34.

Yaacoby T, Schonfeld M and Rubin B (1986). Characteristics of atrazine resistant biotypes of three grass weeds. *Weed Science*, **34**, 181-184.

APPENDIX 1

Baseline data illustrating the induction of GST activity as a result of herbicide treatment.

7.1 The effect of herbicide treatment on protein content and GST specific activity of individual black-grass plants. (For materials and methods, the reader is referred to Chapter 3).

7.1.1. Protein content. Herbicide treatment at recommended FR increased protein content g^{-1} fwt between controls and treated plants of both populations, as demonstrated in Figure 7.1. with the exception of AC210. Statistical analyses by means of t-tests, testing the null hypothesis of zero mean difference at the 95% confidence level, indicated no significant difference ($P<0.05$) in protein content between the controls of the two populations. With the exception of IPU, values for Herbiseed for each treatment were highly significantly different ($P<0.001$) from the controls. Treated Peldon plants were shown to be all significantly different ($P = 0.05$ or less) from the control plants. Comparisons between the two populations indicated clear significant differences ($P<0.05$) in their responses to FE, CP and FPM with the suggestion that the protein content of Herbiseed was affected to a greater extent by herbicide treatment than Peldon.

Additional analysis was carried out to investigate the large differences observed in Figure 7.1. in an attempt to establish why the Herbiseed population apparently responded more than Peldon to some treatments. This was achieved by identifying whether increased protein content was due to plant desiccation from herbicide treatment or whether there was more to resistance than first thought. To achieve this, dry weight was established on the two remaining plants from each pot from each replicate of the trials for each treatment at FR. Before drying, it was evident that Peldon plants were less desiccated than Herbiseed

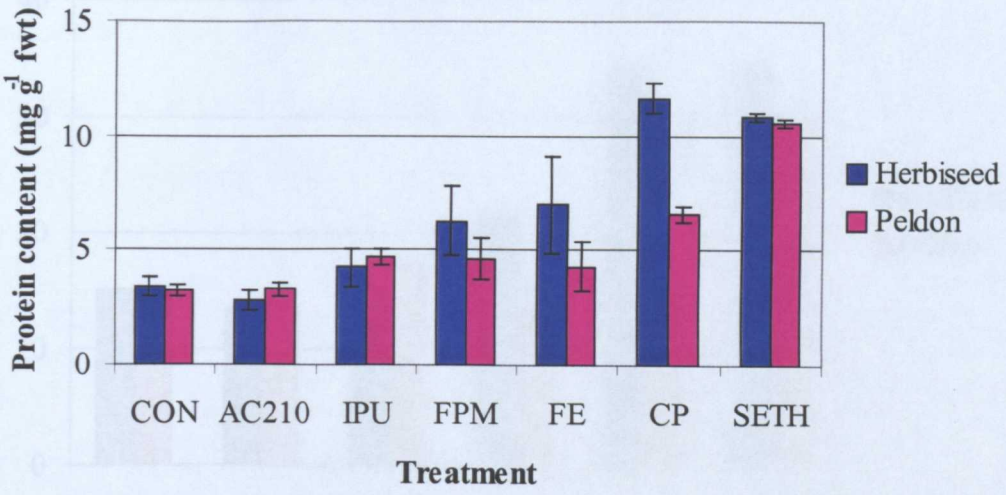


Figure 7.1. Effect of herbicide treatment at FR on mean protein content (mg g^{-1} fwt) of resistant and susceptible black-grass plants, 14 dat. Values are means ±SE values, where $n = 24$.

plants. The plants were oven-dried at 80°C for 48 h and dry weights used to calculate dry weight as a percentage of fresh weight, as shown in Figure 7.2. From this, it was clear that FE, CP and SETH desiccated Herbiseed plants and that SETH also desiccated Peldon plants at FR. The dried plants were then analysed for protein content as described in Chapter 3 and these values are illustrated in Figure 7.3.

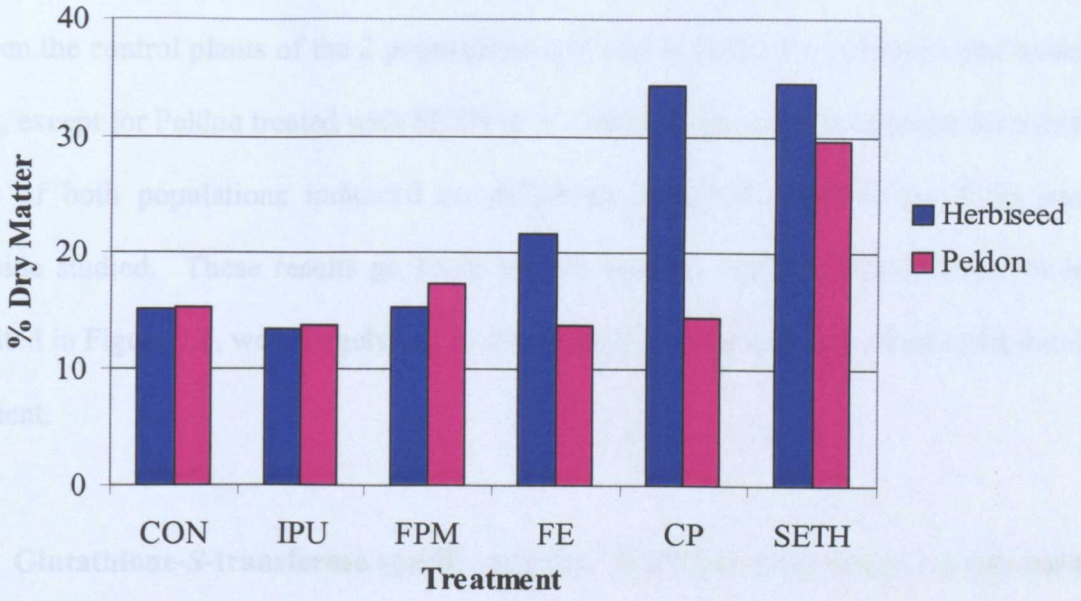


Figure 7.2. Dry matter content of resistant and susceptible black-grass plants expressed as a percentage of their original fresh weight (g) values. Values are means, where $n = 16$.

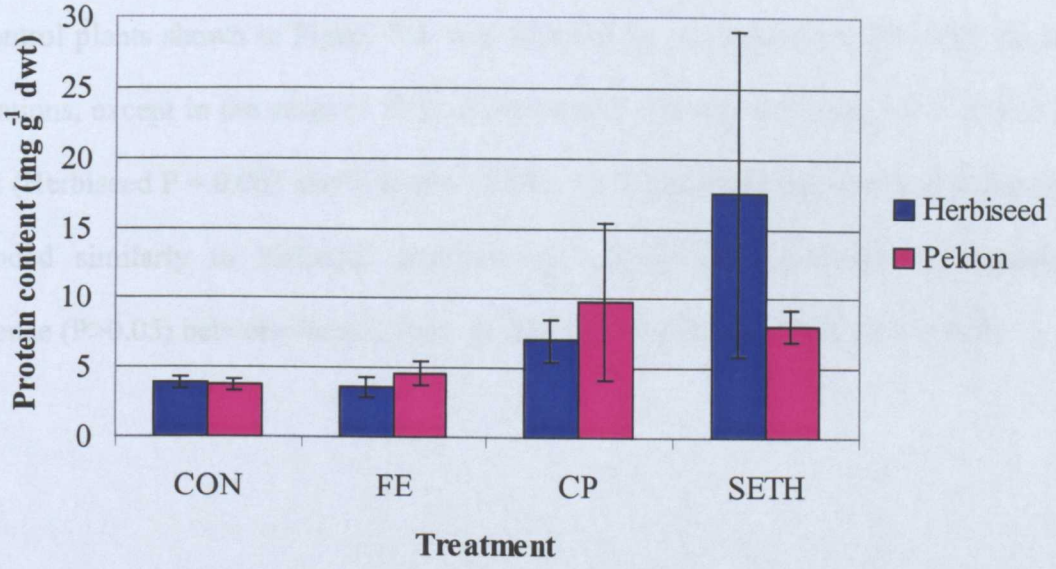


Figure 7.3. Effect of herbicide treatment at recommended FR on mean dry matter protein content (mg g^{-1} dwt) in resistant and susceptible black-grass, 14 dat. Values are means ±SE values, where $n = 16$.

Statistical analysis of these values by t-tests indicated no significant difference (P>0.05) between the control plants of the 2 populations and also between the untreated and treated plants, except for Peldon treated with SETH (P = 0.003). Comparisons between the treated plants of both populations indicated no difference (P>0.05) between the 2 for each herbicide studied. These results go some way to confirm that the large protein values exhibited in Figure 7.1. were largely due to desiccation of the plants as a result of herbicide treatment.

7.1.2. Glutathione-*S*-transferase specific activity. Herbicide treatment at recommended FR elevated GST specific activity between control and treated plants of both populations, as illustrated in Figure 7.4. Statistical analysis by means of t-tests indicated no significant difference (P>0.05) in GST specific activity between the controls of the two populations. Further t-tests were carried out to assess the effect of herbicide treatment on enzyme activity in and between the two populations. The elevation in specific activity over that of the control plants shown in Figure 7.4. was found to be not significant (P>0.05) for both populations, except in the cases of FPM (Herbiseed P = 0.012 and Peldon P = 0.002) and SETH (Herbiseed P = 0.003 and Peldon P<0.001). It is postulated that the two populations responded similarly to herbicide treatment as comparisons indicated no significant difference (P>0.05) between them, except for IPU (P = 0.003) and FPM (P = 0.007).

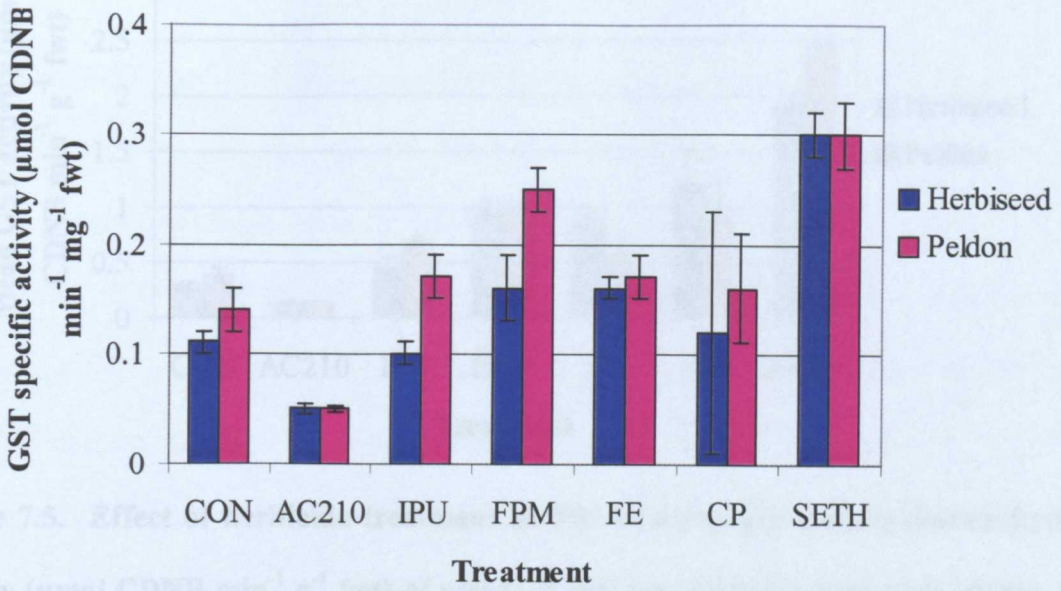


Figure 7.4. Effect of herbicide treatment at FR on mean glutathione *S*-transferase specific activity (µmol CDNB min^{-1} mg^{-1} total protein) of resistant and susceptible black-grass plants, 14 dat. Values are means ±SE values, where $n = 24$.

7.2. Effect of herbicide treatment on endogenous GST activity.

Herbicide treatment at recommended FR elevated GST activity between control and treated plants of both populations, as illustrated in Figure 7.5. Statistical analysis by means of t-tests indicated a significant difference ($P = 0.045$) in GST activity between the two controls. Further analysis indicated that except for FR applications of IPU and AC210 applied to Herbiseed, there were significant differences ($P<0.003$) between the controls and treated plants of both populations for every herbicide applied. There were significant differences ($P<0.05$) between the two populations for IPU and FE, otherwise there were no differences ($P>0.05$) between the response of the two populations and they could again be said to respond similarly to the herbicides applied.

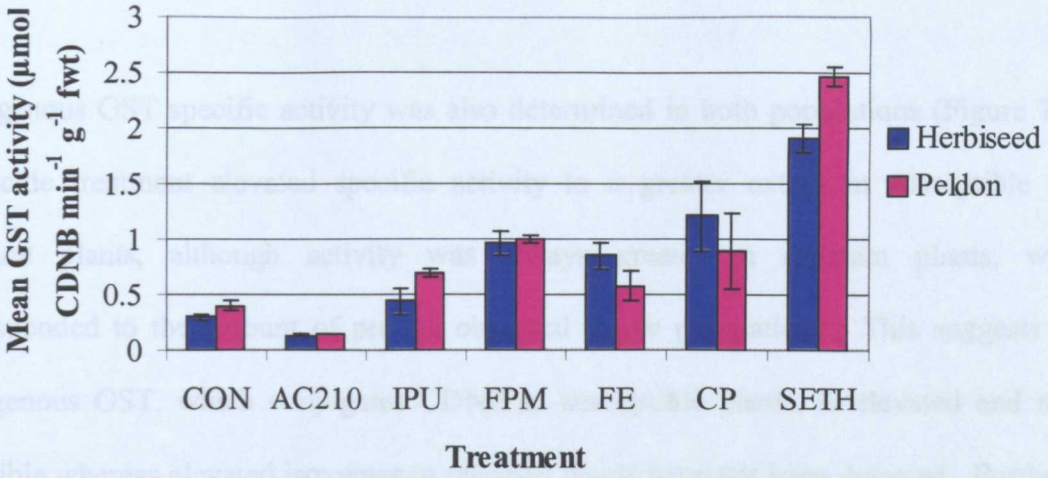


Figure 7.5. Effect of herbicide treatment at FR on mean glutathione *S*-transferase activity (µmol CDNB min^{-1} g^{-1} fwt) of resistant and susceptible black-grass plants, 14 dat. Values are means ±SE values, where $n = 24$.

7.3. DISCUSSION

This study assessed the effect of herbicide treatment on the Herbiseed and Peldon populations. The results indicated that herbicide treatment induced elevations in GST activity (Figures 7.4. and 7.5.) in both populations, with a few exceptions. These were novel observations as few studies have been carried out to assess the effect of these particular herbicides on protein turnover and GST activity.

The effect of herbicide treatment on protein turnover was initially studied. Preliminary results indicated that every treatment induced protein synthesis in both populations, except for IPU and AC210 applied to Herbiseed (Figure 7.1.). However, subsequent analysis indicated that neither protein synthesis nor degradation was induced by herbicide treatment in either population. The observed increases in protein content were confirmed by dry weight analysis to be due to herbicide treatment promoting desiccation in treated plants (Figures 7.2. and 7.3.) and thus concentrated protein content whilst untreated plants

contained more water.

Endogenous GST specific activity was also determined in both populations (Figure 7.4.). Herbicide treatment elevated specific activity to a greater extent in susceptible than resistant plants, although activity was always greater in resistant plants, which corresponded to the amount of protein observed in the populations. This suggests that endogenous GST, which conjugates CDNB in susceptible plants, is elevated and more inducible whereas elevated isozymes in resistant plants have not been detected. Further to this, the effect of herbicide treatment on GST activity g^{-1} fwt of the two populations was studied. Significant differences ($P<0.05$) between GST activity expressed by the control resistant and susceptible populations were observed confirming the observations of Sharples *et al.*, (1995) and Reade *et al.*, (1997) that Peldon demonstrates higher activity as illustrated in Figure 7.5. Herbicide treated plants from both populations demonstrated elevated GST activity over those of the control plants indicating that herbicide treatment induced GST activity, confirming the observations of Reade *et al.*, (1999). There was also a clear correlation between GST activity and herbicide injury as illustrated by Hatton *et al.*, (1996) and Cummins *et al.*, (1997a). It may be that herbicide treatment in this study induced GST subunits in both populations, which are not constitutively expressed in response to the stress of herbicide treatment and the effect of varying environmental conditions within the glasshouse.

It is widely acknowledged that GSTs play important roles in both cellular metabolism and the detoxification of xenobiotics (Marrs, 1996). GSTs have been widely studied with respect to herbicide metabolism, detoxification and resistance. It is well documented that resistant black-grass populations exhibit twice the GST activity of susceptible populations and that they do not require herbicide treatment to do this (Sharples *et al.*, 1995; Reade *et al.*, 1997). This study confirmed that GSTs are induced by herbicide treatment and thus

are involved in herbicide detoxification providing an intrinsic protection mechanism affording herbicide tolerance, which was observed as elevated activity in the Peldon population (Figures 7.4. and 7.5.).

7.4. CONCLUSION

Herbicide resistance was characterised in the susceptible black-grass population Herbiseed and resistant population Peldon by means of biochemical analyses. Novel observations of endogenous GST activity induced by herbicides which have not been previously studied have further confirmed that GSTs play a role in herbicide resistance in black-grass.

APPENDIX 2

EXAMPLES OF ORIGINAL DATA AND STATISTICAL ANALYSES

KEY: d.f. – degrees of freedom; s.s. – sum of squares; m.s. – means of squares; v.r. – variance; F pr – F probability and s.e.d. – standard error of difference.

Raw data used to carry out dose response analysis by analysis of variance of three black-grass populations treated with sethoxydim.

Population	Rate of sethoxydim	Yield (g)	Rep
Rothamsted	1.0	0.190	1
Rothamsted	1.0	0.201	2
Rothamsted	1.0	0.520	3
Rothamsted	1.0	0.510	4
Rothamsted	1.0	0.430	5
Rothamsted	1.0	0.234	6
Rothamsted	1.0	0.206	7
Rothamsted	1.0	0.225	8
Rothamsted	0.5	0.374	1
Rothamsted	0.5	0.233	2
Rothamsted	0.5	0.522	3
Rothamsted	0.5	0.335	4
Rothamsted	0.5	0.720	5
Rothamsted	0.5	0.389	6
Rothamsted	0.5	0.184	7
Rothamsted	0.5	0.289	8
Rothamsted	0.1	8.527	1
Rothamsted	0.1	5.927	2
Rothamsted	0.1	2.953	3
Rothamsted	0.1	3.288	4
Rothamsted	0.1	5.206	5
Rothamsted	0.1	4.971	6
Rothamsted	0.1	2.808	7
Rothamsted	0.1	4.875	8
Herbiseed	1.0	0.619	1
Herbiseed	1.0	0.580	2
Herbiseed	1.0	0.483	3
Herbiseed	1.0	0.514	4
Herbiseed	1.0	0.746	5
Herbiseed	1.0	0.667	6
Herbiseed	1.0	0.414	7
Herbiseed	1.0	0.255	8
Herbiseed	0.5	0.344	1
Herbiseed	0.5	0.504	2
Herbiseed	0.5	0.289	3
Herbiseed	0.5	0.792	4
Herbiseed	0.5	0.815	5
Herbiseed	0.5	0.378	6

Herbiseed	0.5	0.500	7
Herbiseed	0.5	0.387	8
Herbiseed	0.1	7.113	1
Herbiseed	0.1	9.054	2
Herbiseed	0.1	3.718	3
Herbiseed	0.1	4.037	4
Herbiseed	0.1	6.914	5
Herbiseed	0.1	6.423	6
Herbiseed	0.1	9.654	7
Herbiseed	0.1	7.323	8
Peldon	1.0	0.243	1
Peldon	1.0	0.0.445	2
Peldon	1.0	0.364	3
Peldon	1.0	0.514	4
Peldon	1.0	0.586	5
Peldon	1.0	0.376	6
Peldon	1.0	0.234	7
Peldon	1.0	0.294	8
Peldon	0.5	0.381	1
Peldon	0.5	0.411	2
Peldon	0.5	0.848	3
Peldon	0.5	0.421	4
Peldon	0.5	0.266	5
Peldon	0.5	1.082	6
Peldon	0.5	0.189	7
Peldon	0.5	0.319	8
Peldon	0.1	8.085	1
Peldon	0.1	9.224	2
Peldon	0.1	1.512	3
Peldon	0.1	4.729	4
Peldon	0.1	4.611	5
Peldon	0.1	3.283	6
Peldon	0.1	3.291	7
Peldon	0.1	5.116	8

Worked example of analysis of variance

Dose response analysis of three black-grass populations treated with sethoxydim.

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Rep stratum	7	20.484	2.926	1.98	
Population	2	8.180	4.090	2.77	0.071
Rate	2	414.553	207.277	140.37	<0.001
Lin	1	289.782	289.782	196.24	<0.001
Deviations	1	124.771	124.771	84.50	<0.001
Population.Rate	4	11.026	2.756	1.87	0.129
Population.Lin	2	7.069	3.534	2.39	0.101
Deviations	2	3.957	1.979	1.34	0.270
Residual	56	82.692	1.477		
Total	71	536.935			

Tables of means

Grand mean 2.13

Biotype	Rothamsted	Herbiseed	Peldon
	1.84	2.61	1.96

Rate	0.1	0.5	1.0
	5.53	0.46	0.42

Biotype	Rate	0.1	0.5	1.0
Rothamsted		4.82	0.38	0.31
Herbiseed		6.78	0.50	0.53
Peldon		4.98	0.49	0.40

Standard errors of differences of means

	Biotype	Rate	Biotype.Rate
Rep.	24	24	8
d.f.	56	56	56
s.e.d.	0.351	0.351	0.608

Coefficient of variation

Variate: Yield

CV% 26.7

Raw data used to carry t-test analysis between two black-grass populations treated with isoproturon at field rate.

Population	Yield at FR IPU (g)	Rep
Rothamsted	3.035	1
Rothamsted	1.677	2
Rothamsted	1.167	3
Rothamsted	5.809	4
Rothamsted	3.404	5
Rothamsted	2.504	6
Rothamsted	2.231	7
Rothamsted	2.079	8
Peldon	8.308	1
Peldon	7.230	2
Peldon	6.679	3
Peldon	6.440	4
Peldon	8.950	5
Peldon	5.706	6
Peldon	5.216	7
Peldon	4.635	8

Worked example of a t-test

Test for evidence that the distribution means are different between Rothamsted and Peldon treated with IPU at FR.

Sample	Size	Mean	Variance
Rothamsted	8	2.738	2.043
Peldon	8	6.645	2.204

Test statistic t = -5.36 on 14 df.

Probability level (under null hypothesis) p = < 0.001

Raw data used in linear regression analysis to identify relationships in GST activity g^{-1} fwt between individual leaves of harvested black-grass plants.

Plant Number	Leaf Number	Average GST activity (μmol CDNB min^{-1} g^{-1} fwt)
1	1	0.52
2	1	0.71
3	1	0.68
4	1	0.35
5	1	
6	1	0.48
7	1	0.46
8	1	0.90
9	1	0.47
10	1	0.55
1	2	0.16
2	2	
3	2	
4	2	0.19
5	2	
6	2	
7	2	0.57
8	2	0.70
9	2	
10	2	0.26

A blank space indicates that no leaf was present

Worked example of linear regression

Linear regression analysis of GST activity at Site 4 (1999/2000) – First sampling

Response variate: GST activity Missing values - 6

Fitted terms: Constant, Leaf Number

Summary of analysis

	d.f.	s.s.	m.s.	v.r.	F pr.
Regression	1	0.1196	0.1196	3.12	0.103
Residual	12	0.4594	0.0383		
Total	13	0.5790	0.0445		

Percentage variance accounted for 14.0

Standard error of observations is estimated to be 0.196

Estimates of parameters

	Estimate	s.e.	t (12)	t pr
Constant	0.762	0.157	4.85	< 0.001
Leaf Number	-0.193	0.109	-1.77	0.103